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IMMUNE RESPONSE IN MALE GUINEA PIGS INFECTED WITH THE GUINEA PIG INCLUSION CONJUNCTIVITIS AGENT OF *CHLAMYDIA PSITTACI*

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

THOMAS LEE PATTERSON
B.S., Missouri Southern State College, 1979

1994
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This thesis is approved for recommendation to the Graduate Council

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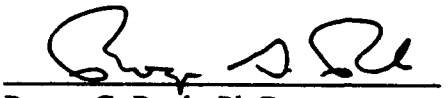

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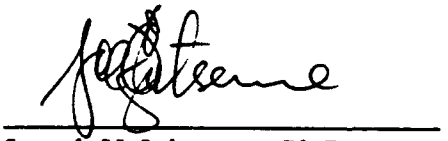

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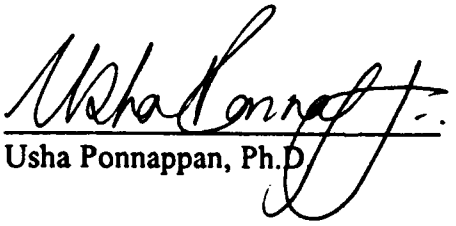

Usha Ponnappan, Ph.D.

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INTRODUCTION

Microorganisms belonging to the family chlamydiaceae are obligate intracellular bacteria. Although once thought to be viruses, members of this family possess a cell envelope similar to that found in gram negative bacteria, contain both DNA and RNA, possess ribosomes, synthesize their own proteins, nucleic acids and lipids, and are sensitive to commonly available antibiotics. Chlamydiae are unique in that they exhibit two morphologically distinct forms; a metabolically inert, small, spherical, infectious elementary body about 0.2 to 0.4 microns in diameter, and a larger, non-infectious, metabolically active intracellular form known as a reticulate body ranging between 0.6 and 1.0 microns in diameter. Because reticulate bodies cannot generate high energy phosphate bonds, they have adapted to the intracellular environment as energy parasites of epithelial cells lining the mucous membranes of birds, mammals, and man. Three different species of chlamydiae are currently recognized: *Chlamydia psittaci, C. pneumonia,* and *C. trachomatis* (20).

While *C. psittaci* and *C. pneumonia* cause respiratory disease in humans, *C. trachomatis* is by far the most significant of the chlamydial pathogens. This species is comprised of 18 serovars designated A through K, including L1, L2, and L3 (50). Repeated ocular infections by *C. trachomatis* serovars A, B, Ba, and C cause a chronic keratoconjunctivitis known as trachoma. This disease is endemic in less developed areas of Africa and the Middle East and is the leading cause of preventable blindness in the world. It is estimated that trachoma afflicts about 500 million people worldwide (20). In the United States however, *C. trachomatis*, for the most part, is sexually transmitted and infects more individuals than all other sexually transmitted diseases combined. Generally, serovars D through K are responsible. It is estimated by the Centers for Disease Control that approximately 4 million Americans are infected annually (42). Carrier rates in the sexually active population are estimated to

range from 5% to 12% (31). Additionally, newborns are at risk of contracting chlamydial conjunctivitis and pneumonia during passage through the birth canal. About 77% of infants born to infected mothers will develop conjunctivitis while 19% will develop pneumonia (5). The three L serovars cause an invasive, systemic chlamydial disease known as lymphogranuloma venereum that is uncommon in the United States (20).

Significant pathology is associated with chlamydial infection of the genital tract in women, although about half of those infected may be asymptomatic (28). Initial symptoms include endocervicitis with a mucopurulent discharge (14). The infection commonly ascends the gential tract, often causing an acute endometritis and salpingitis (27). There is evidence that repeated or chronic infection by *C. trachomatis* may initiate a delayed type hypersensitivity response that results in tubal obstruction (29). This type of immunopathology can ultimately result in ectopic pregnancy or infertility (7).

The pathology associated with chlamydial genital infection in men is milder than that found in women. Although a number of individuals may be asymptomatic (31), many men will develop a nongonoccal urethritis (NGU). Treatment of gonorrhea without consideration of potential chlamydial infection may lead to a postgonoccal urethritis and occasionally epididymitis. About 20% of men infected with gonorrhea will have a concomitant chlamydial infection (15).

Curiously, men show a lower infection rate than women. In one study, Schachter et. al. (44) found that men attending a sexually transmitted disease clinic showed an infection rate of 6.8% while their female contacts had an infection rate of 20%. Because the men in this study had a high prevelance of serum antibody to *C. trachomatis*, there may be an association with serum antibody levels and protection.

It is generally believed that there is some immunity to infection but that it may be short lived as reinfection is common among sexually active individuals. Katz et. al.

(21) examined the relationship between prior sexually transmitted disease and the isolation of *C. trachomatis*. Cultures were collected from 2546 men and 1998 women attending a sexually transmitted disease clinic. Lower rates of chlamydial isolation were reported in both men and women with previously documented chlamydial infection if the infection occurred within the past 6 months. These men showed an isolation rate of only 20% compared with a rate of 40% collected from men with evidence of chlamydial infection greater than 6 months before the index visit of this study. Women exhibited the same pattern with 15% and 37% respectively.

Although humoral immunity is thought to play a key role, the correlation between the immune response and protection from infection is far from clear. In one study by Svensson et. al. (47), serum IgG titer to *C. trachomatis* was found to be inversly proportional to the isolation of the organism. Of 23 women with positive cultures, 12 had low titers of ≤ 64 while only 6 had higher titers between 128 to 256. The remaining 5 had titers greater than 512. In contrast, 26 women with a history of prior infection were negative for chlamydial isolation. Of these, 17 had titers of greater than 512, 4 had titers between 128 to 256 and only 5 had titers of ≤ 64.

Conversly, Brunham et. al. (6) compared the serum IgG levels with quantitative recovery of *C. trachomatis* from the cervix of infected women. No correlation was found. However, an inverse correlation was established when chlamydial isolation was compared to the presence of secretory IgA recovered from endocervical secretions. Women with the lowest number of recovered organisms demonstrated the highest prevelance of secretory IgA antibody. Whereas 63% of women demonstrating secretory IgA showed a recovery of less than 10 inclusion forming units (IFU) per milliliter of transport media; only 21% with secretory IgA showed IFUs greater than 10,000 per milliliter.

Another approach to describe the role of humoral immunity is to examine the chlamydial proteins that are immunogenic. This would help identify those proteins that might elicit a protective antibody response. Newhall et. al. (26) electrophoretically separated the proteins of all 15 serovars of *C. trachomatis* and tested the sera from culture positive individuals by immunoblot assay. All sera reacted with the 60 kDa outer membrane protein (omp2) and the 57 kDa heat shock chlamydial protein. The response to lipopolysaccharide was fairly strong also. In contrast, an antibody response to the 40 kDa major outer mambrane protein (MOMP) was often observed but was usually weak. Oddly, sera from groups of individuals (children and nuns) presumed not to have antibody to *Chlamydia* did not react with the 60 kDa protein but did react weakly with MOMP and a 29 kDa chlamydial protein. This was explained as probably a cross reactive antibody elicited by an immunogen with similar epitopes. Interestingly, the 40 kDa MOMP has generated some interest as it has been determined that antibody to this protein is protective but it does not protect by preventing attachment (11).

Caldwell et. al. (11) demonstrated that MOMP purified from the organism would elicit an antibody that neutralizes homologous strains in a complement independent manner in vitro. Furthermore, it was learned that neutralization was not due to extracellular aggregation of organisms, inhibition of attachment, or prevention of entry into the host cell. Neutralization occured after endocytosis. It was also determined that intact, dimeric IgG antibody was necessary because neutralization was lost after papain digestion of the antibody. These observations suggest that IgG bound to the chlamydial surface facilitates lysosomal fusion or perhaps prevents differentiation into reticulate bodies by inhibiting the transfer of energy rich compounds from the host cell into the organism. Because MOMP has been shown to elicit a protective antibody response, it is currently the center of interest for the development of subunit and recombinant experimental vaccine regimens.

Although the humoral immune response plays an important role in the resolution and protection against chlamydial infections, the cell mediated response must also be considered. In an evaluation of the cell mediated response, Brunham et. al. (8) found that a positive lymphocyte transformation assay was a good indicator of current infection. Lymphocyte stimulation indices from women with recent exposure to non gonococcal urethritits were positively correlated with isolation of *C. trachomatis* from the cervix. There was no such correlation with serum antibody. This is expected as the cell mediated response is characteristically short-lived and decreases rapidly after resolution of infection while serum antibody persists long after the infection has been resolved.

However, the cell mediated response may not be entirely beneficial. There is some evidence that the cell mediated response may be responsible for salpingitis and subsequent scarring of the fallopian tubes upon chronic or recurrent infection. Current thought among some investigators is that the 57 kDa chlamydial heat shock protein is responsible for the pathology (24). Witkin et. al. (52) ran a lymphocyte proliferation assay against the 57 kDa heat shock protein. Peripheral blood mononuclear cells were collected from 18 women diagnosed with salpingitis. Additionally, 42 healthy subjects were tested in the same manner. A total of 9 (50%) of the 18 women with salpingitis showed a positive lymphocyte response to the heat shock protein while only 3 (7%) of the asymptomatic controls showed a positive response. It was also noted that lymphocytes from 10 women suffering from only cervicitis did not respond, indicating that the cell mediated response may be restricted to an ascending infection.

From these studies it can be seen that a genital infection by *C. trachomatis* elicits both humoral and cell mediated immune responses. The precise nature, function, and interplay of each remains to be determined. However, investigations using human subjects need to be carefully considered. In one study, experimental vaccination with

whole organism vaccines accelerated ocular inflammation upon reinoculation (13). Obviously the use of human subjects in experiments of this nature raises ethical questions.

To circumvent this problem, the standard scientific approach has been to define specific biological mechanisms by experimentation with animal models and then to carefully apply that knowledge in the design of human trials. However, a suitable animal model must be chosen. Ideally, the animal should be susceptable to the human pathogen and should exhibit pathology similar to that found in humans . Using this approach, much has been learned about the immune response to *Chlamydia* from experimentation with various animal models.

Some species of non-human primates have been shown to be excellent models. They are readily infected with the human associated serovars of *C. trachomatis* and they develop pathology similar to that found in humans (19) (29). However, the cost and maintenance expenditures for these animals are prohibitive for most studies. Mice are small, inexpensive, and have been used in numerous chlamydial studies. They are easily infected with a biovar of *C. trachomatis* known as mouse pneumonitis agent (MoPn) (2) which can infect the cervix, uterine horns, and ova ducts (48). Guinea pigs have also been used extensively. They are readily infected with a member of *C. psittaci,* the agent of guinea pig inclusion conjunctivitis (GPIC). Although GPIC is a natural parasite of the guinea pig and normally infects ocular tissues, experimental inoculation into the vagina produces cervicitis, endometritis, and salpingitis (1) (38).

This animal has served as a very good model and much has been learned about the immune response to genital infection with *Chlamydia* through experimentation with female guinea pigs. To study the course of a primary chlamydial genital infection and challenge infection, Rank et. al. (35) inoculated female guinea pigs 30, 77, 155, and 294 days after primary genital infection with GPIC. The primary infection was

monitored by examining cells obtained from vaginal scrapings for inclusion bodies and by culture. The highest number of organisms was recovered between days 6 and 9 and cleared by day 21 or 24. Serum and secretory antibodies peaked on day 28 post infection. Of the animals challenged at day 30 , 3 of 5 were completely immune to infection while the other 2 showed strong immunity, shedding only a few organisms on day 3 or 6 post challenge. By day 75 post infection, secretory IgG, IgA, and serum IgG had decreased significantly. However, all guinea pigs challenged on day 77 became reinfected with the infection lasting only 3 to 9 days, much shorter than the 21 days found on the initial infection. The group challenged 155 days post infection demonstrated similar results. The group challenged at 294 days showed an infection that spanned 9 to 18 days. This study suggests that high titers of secretory and serum antibody may be associated with protection but that protection wanes with time as antibody titers decrease.

An additional study by Batteiger and Rank (3) characterized the antigen specific antibody response. Female guinea pigs were experimentally infected by intravaginal inoculation of GPIC. An immunoblot assay was used to evaluate antibody response in both serum and genital secretions. Most animals showed a relatively strong serum antibody response to the chlamydial 60 kDa outer membrane protein (omp2) and lipopolysaccharide. A weaker response was seen to an 84 kDa outer membrane protein and the 39 kDa major outer membrane protein (MOMP). Serum antibodies to omp2 were quite persistant and were detected as long as 813 days post infection. Immunoblot assays of secretory IgA showed a response to omp2, MOMP, and lipopolysaccharide, but not to the 84 kDa outer membrane protein. The investigators noted that the secretory IgA became undectable by day 50 post infection. It is interesting to note that animals challenged 825 days after initial infection still demonstrated some degree of immunity. Although the challenge inoculation

produced infection, the period of chlamydial shedding was significantly shorter and less intense than the primary infection.

In another study, Rank et. al. (40) investigated the role of humoral immunity in the resolution of infection by selectively suppressing the B cell population alone or the B cell and T cell populations with varying dose regimens of cyclophosphamide. Daily treatment with low doses suppressed both humoral and cell mediated responses while high single doses at 9 day intervals suppressed only humoral immunity. Although a genital infection in female guinea pigs usually resolves within 21 days, those animals with suppression of both humoral and cell mediated responses showed no signs of recovery even to day 36 at which time the last animal of the group died. When humoral immunity alone was suppressed, a persistant infection was seen until day 57 by which time all animals had died. Another group of animals treated with smaller doses of cyclophosphamide reached peak serum IgG titers at day 42 post infection. This peak correlated with resolution of the infection. This study suggests that the cell mediated response alone could not resolve the infection and that humoral immunity was necessary.

A passive immunization study by Watson et. al. (51) showed that administration of immune serum provided no protection against ocular infection. However, Rank and Batteiger showed a significant degree of protection in the genital tract by passive transfer of serum antibody (34). Female guinea pigs were injected intraperitoneally with pooled immunoglobulin to GPIC. The guinea pigs were then challenged intravaginally with live GPIC and monitored for infection. Interestingly, the animals showed a significant degree of protection in the genital tract. Although the duration of infection was similar to that of untreated controls (20 days), the intensity of infection was markedly decreased. Approximatley 70% fewer inclusions were detected in the treated animals. Immediately prior to infection, high titers of GPIC specific IgG were present in the serum and cervical secretions indicating that the

antibody had transudated into the lumen of the genital tract. In contrast, Watson could not detect the presence of passively administered antibody in ocular secretions. The study by Rank et. al (34) strongly suggests that serum antibody may provide at least partial protection against genital chlamydial infection.

Thus it has been established that humoral immunity plays a key role in the response to genital infection by *Chlamydia.* As previously mentioned, the cell mediated response alone was unable to resolve the infection (40), but that does not rule out the possibility that this response, in concert with the humoral immune response, is necessary for resolution or protection.

In an effort to examine the cell mediated response, Rank and Barron (32) injected female guinea pigs with anti-thymocyte serum (ATS) to attenuate the cell mediated response. The animals were then inoculated intravaginally with GPIC. Even though delayed hypersensitivity responses to oxazolone and heat inactivated GPIC were suppressed, serum antibody rose to normal levels indicating that perhaps the T-helper cell population was not affected by ATS. Curiously, the appearance of secretory IgA was significantly delayed in the treated animals. Untreated animals developed secretory IgA by day 19 while the ATS treated animals did not develop IgA until day 33 post infection. Additionally, the IgA titers were lower in the treated animals when compared to the controls. The net effect was that the animals receiving ATS had a prolonged infection. Control animals resolved the infection by day 20 while the treated animals required 45 to 50 days to clear the infection. This study suggests that the cell mediated response is also an important factor in immunity to *Chlamydia.*

In a similar study, Rank et. al. (39) infected female guinea pigs intravaginally and challenged different groups at 30 days and 75 days post infection. Prior to challenge the animals were treated with ATS. At the 30 day challenge 70% of the ATS treated animals became reinfected compared to 40% of the untreated controls. All animals challenged at 75 days post infection became reinfected but the ATS treated group

showed an infection similar in duration to that of the initial infection. The untreated control group resolved the infection by day 12 post challenge. It was noted that the intensity of infection for all groups was less than that found in the initial infection. To confirm the effect of ATS treatment, the cell mediated response was evaluated by proliferative response of peripheral blood mononuclear cells to GPIC antigen. Those groups treated with ATS showed no proliferative response while the control groups demonstrated a strong response. Antibody levels in both serum and secretions were also measured. Again, ATS treated animals developed a normal serum antibody response but in this case, the secretory IgA also showed a strong response similar to that of the untreated controls. This study demonstrated that the cell mediated immune response is essential for resolution of a challenge infection.

The precise mechanism of the cell mediated responose to *Chlamydia* has not been elucidated but it appears that cytotoxic lymphocytes are not involved (30). Igietseme and Rank found that GPIC antigen-specific T cells isolated from the genital tract of infected female guinea pigs play a significant role in resolution of infection and are probably responsible for resistance to reinfection (17). Antigen-specific T cell activity was high 30 days after initial infection and correlated with a marked resistance to reinfection. However, at 75 days post infection, GPIC specific T cell activity had decreased and all animals challenged became reinfected. Additionally, two in vitro studies suggest that gamma interferon (IFN-γ) produced by T-lymphocytes may be responsible for inhibition of the growth of intracellular *Chlamydia* and the subsequent killing of infected cells (9) (10). It has also been found that mice depleted of functional IFN-γ by monoclonal antibody had significantly prolonged gential infections when inoculated intravaginally with MoPn biovar of *C. trachomatis* (37). This study also investigated passive administration of recombinant IFN-γ. A total of 6 nude (athymic) mice were given recombinant IFN-γ. Three resolved the infection while the others had lower numbers of recovered

inclusion forming units when compared to the nude controls that did not receive IFN-γ. None of the control nude mice resolved the infection. It has been found that tumor necrosis factor alpha (TNF-α) also plays a key role in the cell mediated response and there is evidence that it may act synergistically with IFN-γ (45).

Generally, the emerging picture is that both antibody and the cell mediated response are necessary for resolution of and resistance to infection. The logical question then is whether protection can be induced by immunization.

Rank et. al. (36) immunized female guinea pigs with viable or ultra violet (UV) light inactivated GPIC by intravenous, subcutaneous, oral, or ocular routes and then challenged them with intravaginal inoculation of viable GPIC. Those guinea pigs immunized with UV inactivated GPIC showed a decrease in intensity of challenge infection but no increase in resistance to infection or abbreviation of the course of infection. There was a significant lack of protection in those animals immunized orally with inactivated antigen. Much better protection was acheived by intravenous and subcutaneous injection of viable GPIC. From 20% to 40% of those animals were completely resistant to infection. Animals receiving live GPIC by the oral route were not resistant to infection but did show a shorter span of infection than did the unimmunized control group. Overall, it was found that immunization with viable organisms produced a stronger protective immune response than immunization with inactivated antigen. The reasons for this are not apparent. Measured immune responses were generally similar in groups receiving live or inactivated chlamydial immunizations. Perhaps different antigens expressed only by the dividing organism may elicit a qualitatively different T cell immune response that provides somewhat better protection. This has been shown to be the case with protective T cell clones elicited only by live *Leishmania* organisms (25). In addition, recent studies have shown that some chlamydial antigens are present only in metabolically active *C. psittaci* (43).

Whatever the mechanism, it appears that complete, long-term protection from *Chlamydia* may not be possible at this point, especially since the best immunizing event, an actual genital infection, only provides short-term protection. A more reasonable goal might be to lessen the severity and duration of infection by artificial manipulation of the immune response through immunization. Since the ascending infection is associated with pathology in the female, another approach might be to confine the infection to the lower genital tract. Still another avenue of investigation is to try to reduce the inoculum size by immunization of the male. As mentioned previously, men attending a sexually transmitted disease clinic were found to have a much lower infection rate than their female partners (44). If males are less susceptable to infection or reinfection, it may be due to differences in the immune response or differences in anatomy of the genital tract or a combination of both. Investigations along these lines might prove fruitful for if males respond significantly better to immunization, the resulting infection rate in women would potentially decrease.

Understandably, there has been a relatively small amount of research on the male immune response to *Chlamydia* for it is the female that exhibits most of the pathology. Using the male rat as an animal model, Jantos et. al. (18) examined the course of chlamydial induced epididymitis. When *C. trachomatis,* biovar MoPn, was injected into the vas deferens, the organism could be recovered from the epididymides for up to 90 days. By day 30, the organism was found in the testes. The epididymides were enlarged showing pyogranulomatous inflammation. There was also testicular atrophy with significant loss of the germinal epithelium. Serum IgM and IgG levels were monitored for 90 days post infection. IgM antibodies to *C. trachomatis* were evident by day 5 and peaked at day 30 after which the level dropped significantly. The IgG levels at day 5 were much lower but rose dramatically and also peaked at day 30. This level however did not drop significantly

and remained fairly elevated throughout the experiment. The animals in this study were not challenged with an additional infection.

A similar study was done by Moller and Mardh using male grivet monkeys (23). A suspension of *C. trachomatis* was injected into the left spermatic cord of two monkeys and again, significant pathology was associated with the epididymides. After 7 days, the left testis, epididymis, and spermatic cord were removed from one monkey for histological examination. After 14 days, the same procedure was performed on the other monkey. The histological findings of both monkeys were essentially the same. The lumina of the ducts of the eipidymides were filled with an exudate containing lymphocytes and polymorphonuclear leukocytes. Inflammatory cells were also present in the testes and spermatic cords. One monkey developed a serum IgG titer of 16 on day 42 post infection while the other monkey had a titer of 64. By day 75, no antibodies could be detected in either monkey. It must be noted that the infected tissues were excised early in the infection and this may have had a significant effect on the antibody response.

Digiacomo et. al. (12) investigated urethral infections in baboons. Two adult male baboons were infected with *C. trachomatis*, serovar D, by urethral catheter. Immune responses were evaluated by serum IgG titer. One baboon had a peak titer of 512 at 55 days post infection while the other had a peak titer of 64 at 35 days. Surprisingly, challenge infections did not boost the titers. The course of infection was monitored by culturing organisms from urethral swabs. One baboon shed organisms for 90 days and the other for 96 days. The numbers of inclusion forming units peaked at 4×10^4 on day 25 in one baboon and at 9.1×10^4 on day 35 in the other. Both animals were challenged with a second inoculation 139 days post infection. One of the animals could not be reinfected and the other had only 1 positive culture of 10 inclusion forming units on day 3 post challenge.

Cystitis in male guinea pigs was also investigated (41). Animals were infected by urethral injection of GPIC or by placing a drop of the suspension on the meatus of the extruded penis. Urethritis and cystitis developed in animals infected by both methods indicating that *Chlamydia* can cause an ascending infection in males. The urethral infection followed the same duration as that found in female guinea pigs, clearing in approximatley 18 to 20 days. However, animals immunosuppressed with cyclophosphamide treatment showed a prolonged infection of up to 50 days. Cystitis was associated with immunosuppressed animals but one control animal did develop cystitis. To assess immunity, serum IgG titers were monitored during the course of infection. Immunosuppressed animals showed titers that were severely depressed and their infections were significantly prolonged. Again, this study illustrates the importance of the humoral immune response in chlamydial infections.

Although these studies are revealing, much about the male immune response to *Chlamydia* is still unknown. Determining the nature of the male immune response might prove instrumental in the development of an effective or partially effective vaccine. It follows that the choice of an appropriate animal model is important. The male guinea pig is a logical choice as there is a solid base of data on the female guinea pig that is available for comparison. A basic study of the immune response in male guinea pigs is required; more specifically, (1) the quantitation of the course of chlamydial genital infection in the male needs to be elucidated, (2) the humoral and cell mediated response must be described in greater detail, (3) resistance to challenge infection in males must be evaluated and compared to that of females, and (4) the male response to immunization must also be evaluated for comparison. Therefore, it is the purpose of this investigation to address these issues by evaluating immune parameters in male guinea pigs infected with *Chlamydia.*

MATERIALS AND METHODS

Experimental Animals

Male Hartley strain guinea pigs weighing 450-500 grams were obtained from Sasco Co., Omaha, Nebraska. Upon arrival, all animals were banded on the right ear and housed in individual cages covered with fiberglass filters in the animal section of the Biomedical Research Center, University of Arkansas for Medical Sciences. Animals received food and water *ad libitum* and were maintained on a 12:12 hour light:dark cycle.

Preparation of GPIC Stock

Stock GPIC grown in HeLa cell culture was thawed and kept on ice. The stock was diluted in warm (37C) cyclohexamide medium (10% fetal calf serum, 3.0 mg glucose/ml, 100 ug vancomycin/ml, 100 ug gentamicin/ml, 1.25 ug fungizone/ml, 0.5 ug cyclohexamide/ml, in Eagle's Minimal Essential Media, Gibco BRL, Grand Island, New York) to a concentration of 3 to 4 X 10^8 inclusion forming units per ml so that approximately 80-100% of HeLa cells were infected at 40 hours. Media was removed from fresh HeLa cell culture grown in 6 to 8 75 cm^2 tissue culture flasks (Becton Dickson, Plymouth, England). One ml of the diluted chlamydiae stock was placed in each flask and incubated at room temperature for 3 hours with intermediate agitation. After incubation, 15-20 mls of warm (37C) cyclohexamide media was added and flasks were incubated at 37C for 40 hours. The monolayer was scraped and the contents of all flasks were pooled in 50 ml conical centrifuge tubes and held on ice. The suspension was sonicated with a Fisher Sonic Dismembrator Model 300 for 60 seconds. The suspension was then centrifuged at 500 x g in a Beckman TJ-6 centrifuge for 10 minutes at 4C. Supernate was removed and placed in a sterile 50 ml high speed centrifuge tube.

The supernate was centrifuged in a Sorval RC-2B at 30,000 x g for 30 minutes at 4C. The pellet was resuspended in 15 mls of sucrose phosphate buffer (0.200 M sucrose, 0.012 M K_2HPO_4, 0.008 M KH_2PO_4), divided into 1 ml aliquots, and stored at -70C until needed. One ml was reserved to titer the number of IFUs in the suspension by serial dilution. Titers were determined before use of the stock by infecting HeLa cell cultures.

Infection of Guinea Pigs with GPIC

Animals were infected by placing 2 X 10^6 inclusion forming units (IFU) suspended in 20 ul of sucrose potassium glutamate buffer (0.220 M sucrose, 0.0038 M KH_2PO_4, 0.0086 M Na_2HPO_4, 0.0045 M L-glutamic acid) on the tip of the urethra of the extruded penis. The penis was allowed to retract normally.

Preparation of Antigen for Immunization

Antigen for immunization was prepared by inactivating GPIC by ultra violet (UV) light exposure. Stock HeLa-grown GPIC, 500 ug of protein/ml, was thawed and placed in a 35 X 10 mm petri dish with a 7mm X 2mm micro stir bar. The dish was positioned on a magnetic stirrer which was approximately 3-4 cm from a Westinghouse Sterilamp G36T6L. The suspension was exposed for 3 hours. Inactivation was verified by lack of infectivity upon culture of the suspension.

Immunization

The UV inactivated GPIC was drawn into a sterile 1 ml syringe and each animal was injected subcutaneously at 2 different sites in the inguinal area with 50 ul at each site for

a total of 100 ul of UV inactivated GPIC. Animals were immunized initially and were boosted 2 times, once at 2 weeks and then at 4 weeks post inoculation using the same method.

Assessment of Infection

To assess the level of infection, guinea pigs were anesthetized by inhalation of methoxyfurane (Pitman Moore, Mundelein, Illinois). A type 1 sterile dacron swab (Spectrum Laboratories, Houston, Texas) was inserted approximately 1 to 2 cm into the urethra and rotated several times to collected a sample of epithelial cells. The swab was placed immediately in 1 ml of cold transport media (3% fetal calf serum, 100 ug vancomycin/ml, 100 ug gentamicin/ml, 1.25 ug fungizone/ml in sucrose phosphate buffer) and stored at -70C.

Culture specimens were thawed and immediately vortexed 1 minute with 2 sterile glass beads. After vortexing, 1.3 ml of cyclohexamide media was added and specimens were mixed. One ml of the specimen was pipetted onto a monolayer of fresh McCoy cells grown on a 12 mm diameter circular glass coverslip at the bottom of a glass shell vial. The vial was tightly capped with a sterile rubber stopper. All vials were centrifuged at 1300 x g at 28C for 1 hour. Specimens were then incubated at 37C for 2 hours at which time the cyclohexamide media was replaced. All specimens were then incubated 24 hours at 37C. Media was suctioned off and 1 ml of acetone was added to each specimen. All vials were incubated at room temperature for 2 hours. The acetone was removed and specimens were then gently washed 3 times with phosphate buffered saline (0.28 M NaCl, 0.005 M KCl, 0.004 M Na_2HPO_4, 0.001 M KH_2PO_4). Plasma from immune guinea pigs was diluted 1:100 in phosphate buffered saline (PBS) and 0.25 ml of this dilution was added to each vial and incubated for 1 hour at 37C. Cell monolayers were gently washed 3 times with PBS. Fluorescein conjugated goat anti-

guinea pig IgG (Organon Teknika, Westchester, Pennsylvania) was diluted 1:80 in PBS and 0.25 ml were added to each vial. All specimens were incubated for 1 hour at 37C. Specimens were then washed 3 times with PBS and counter stained with Evan's Blue (Aldrich Chemical Co., Milwaukee, Wisconson) for 10 minutes at room temperature. The stain was removed and specimens were washed 2 times with PBS. The last wash was removed and 1 ml of H_2O was added. Coverslips were gently removed with a needle and forceps and allowed to dry at room temperature, cell monolayer facing up. Coverslips were then placed, cell monolayer down, on a drop of glycerol on a glass microscope slide. Slides were read on a Zeiss Model XBO 75W/2 fluorescent microscope under the 40X objective. The number of IFUs in 20 fields was counted and multiplied by 81.8 to correct for the entire area of the coverslip. This was considered to give the number of IFUs per swab.

Collection of Plasma

Plasma was collected using the method of Lopez and Navia (22). Guinea pigs were anesthetized with 0.1 cc of Innovar-Vet (Pitman-Moore, Mundelein, Illinois) intramuscularly. The lateral saphenous vein was punctured with a lancet and the blood was collected in a heparinized Caraway capillary tube (Fisher Scientific, St. Louis, Missouri). Whole blood samples were transferred to a 1ml plastic centriguge tube and centrifuged 2 minutes at 4000 x g in a Fisher Model 59 table-top centrifuge. Plasma was removed and stored at -20C.

Preparation of Antigen for Enzyme Linked Immunosorbent Assay

Fresh McCoy cells were infected with GPIC stock in 6 to 8 150 cm^2 tissue culture flasks. Cells were pooled in 50 ml conical centrifuge tubes and sonicated in a Fisher

Sonic Dismembrator for 45 seconds. The tubes were then centrifuged at 192 x g in a Beckman T5-6 at 4C for 10 minutes. Supernates were poured into sterile 50 ml high-speed centrifuge tubes and centrifuged in a Sorvall RC-2B at 30,900 x g for 30 minutes at 4 C. Supernates were discarded and pellets were combined in 6 mls of Hank's Balanced Salt Solution (Hank's 10X, Gibco, Grand Island , New York, diluted 1:10 in H_2O). Pellets were resuspended in 3 ml of Hank's Balanced Salt solution (HBSS) with 0.36 ml of DNAase/RNAase solution (8 mg DNAase I, 72 mg RNAase A, Sigma, St. Louis, Missouri, in 4ml HBSS). The suspension was incubated 1 hour at 37C and was sonicated for 4 seconds. The gradient was prepared in 2 Beckman ultra-clear 14 X 95 mm centrifuge tubes with a 10 cm trochar on a 5 ml syringe. HEPES buffer (0.01 M HEPES, 0.15 M NaCl in H_20) was prepared. Solutions were carefully layered starting with the GPIC suspension. Next, 2.5 ml of a 40% renografin solution (1ml Angiovist, Winthrop Pharmaceuticals, New York, New York, 0.25 ml HEPES buffer, 1.25 ml H_2O) was added to each tube. Then 5 ml of a 44% renografin solution (2.2ml Angiovist, 0.5 ml HEPES buffer, 2.3 ml H_2O) was added. The final solution, 2.5 ml of a 54% renografin layer (1.35 ml Angiovist, 0.25 ml HEPES buffer, 0.9 ml H_2O) was added and the gradient was centrifuged in a Beckman L5-50B ultracentrifuge at 43,000 x g at 4C for 1 hour. The layer containing the elementary bodies between the 44% and 54% gradient was removed along with about two thirds of the 44% layer with a sterile Pasteur pipet. This was placed in a 15 ml high-speed centrifuge tube and centrifuged in a Sorval RC-2B centrifuge at 30,900 x g for 30 minutes at 4C. The supernate was discarded and the pellet was resuspended in 6 ml of PBS. This centrifugation procedure was repeated 2 more times. After the final centrifugation, the pellet was resuspended in 2 ml of PBS.

The amount of protein in the antigen was determined by the Lowry Method. Glass culture tubes, 12 x 75 mm, were numbered 1 through 8. Bovine serum albumin (Sigma, St. Louis, Missouri) was added in increasing concentrations to tubes 2 through 7 to create a standard curve ranging from 20 ug of protein to 100 ug of protein. Tube 1 only

received 0.4 ml normal saline, 2 ml of protein reagent (0.10 M NaOH, 0.07 M Na_2CO_3, 0.06 M $CuSO_4$, 0.09 M Na tartarate) and 0.2 ml phenol reagent (1 N Folin-Ciocalteau) and served as a reagent blank. The GPIC antigen solution, 0.025 ml, was added to tube 8 along with 2 ml of protein reagent and 0.2 ml of phenol reagent. The same amount of protein and phenol reagent was added to the standard protein tubes 2-7. All tubes were incubated at room temperature for 30 minutes. The optical density of all tubes was read at 600 nm on a Hitachi spectrophotometer. The optical density of the antigen in tube 8 was compared to the standard curve to determine the protein concentration. Sterile SPG was added to bring the antigen concentration to 500 ug of protein/ml. Antigen was stored in 1 ml aliquots at -70C until use.

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) has been described previously (33). This assay was done to determine antibody levels to GPIC in plasma. Antigen was prepared by diluting 0.1 ml of McCoy grown GPIC (500 ug/ml) in 9.9 ml of 0.5 M $NaHCO_3$. Using an octapet, 0.1 ml of this dilution was pipetted into each well of a 96 U-shaped well microtiter plate (Linbro Titer-Tek, Aurora, Ohio). Plates were incubated at room temperature, overnight in a moisture chamber. Wells were emptied and washed 2 times with PBS Tween (0.28 M NaCl, 0.001 M KH_2PO_4, 0.004 M NH_2PO_4, 0.005 M KCl, 0.55 mg/ml Tween 20, Malincrodt Chemical Co., Paris, Kentucky) with 5%-bovine serum. After the second rinse, the wells were filled with PBS Tween-5% bovine serum, covered, and incubated for 1 hour at 37C. Wells were emptied and 200 ul of PBS Tween-2% bovine serum was added to the first row using a 100 ul octapet. All additional wells were filled with 100 ul of PBS Tween-2% bovine serum. Then, 20 ul of the PBS Tween-2% bovine serum was removed from each well of the first row. Individual samples of thawed plasma, 20 ul, were added to each well of the first row.

Octapet tips were placed in the first row and the specimens were carefully mixed by drawing up and expelling the diluted specimen several times. After mixing, 100 ul of specimen was delivered into the next row of wells. This row was mixed in a similar manner as was each consecutive row through row 11. Then 100 ul was drawn out of the 11th row and discarded. Nothing was added to the 12th row as this served as a blank. The plate was then covered and incubated for 1 hour at 37C. All wells were washed 3 times with PBS Tween-2% bovine serum. Peroxidase labeled rabbit anti-guinea pig IgG (ICN Immunobiologicals, Lisle, Illinois) was diluted 1:4,000 in PBS Tween-2% bovine serum and 100 ul of this was added to each well. The plate was again covered and incubated for 1 hour at 37C. After incubation, the wells were washed 3 times with PBS Tween-2% bovine serum. Then 100 ul of 0.5% H_2O_2 was diluted in 10 ml of a 10% solution of 5-amino salicylic acid (Sigma, St. Louis, Missouri) and 100 ul of this dilution was added to each well. Plates were sealed with tape and incubated in the dark, overnight. The optical density was read by a Biotek Instruments Microplate Autoreader, Model EL311 at 494 nm. Optical density readings of less than 0.100 were considered negative. The titer was recorded as the reciprocal of the dilution of the highest titered positive well.

Peripheral Blood Mononuclear Cell Collection

Guinea pigs were anesthetized by inhalation of methoxyflurane. A 22 gauge, 1.5 inch sterile hypodermic needle was attached to a 5 ml sterile syringe containing 0.4 ml of acid-citrate-dextrose anticoagulant (44 mg tri-sodium citrate/ml, 16 mg citric acid/ml, 50 mg dextrose/ml). The animal was positioned ventral surface up and the thoracic area was swabbed with 70% ethanol. The needle was inserted at the left sternal notch and angled toward the center of the chest to puncture the heart. Then 3 to 4 ml of blood was aspirated and the needle was quickly withdrawn.

Peripheral Blood Mononuclear Cell Transformation Assay

The assay for the antigen specific transformation of peripheral blood mononuclear cells has been previously described (39). Blood collected from cardiac puncture was transferred to a sterile 15 ml conical centrifuge tube. Five ml of RPMI 1640 medium modified with L-glutamine (MediaTech, Washington, D.C.) was added to each specimen and gently mixed. Three ml of Histopaque (Sigma Co., St. Louis, Missouri) was carefully layered under the cell/media mixture. Specimens were centrifuged for 40 minutes at 200 x g at 20C in a Beckman GPR table top centrifuge. A sterile 5 inch Pasteur pipette was used to draw up the buffy coat containing the blood mononuclear cells. The buffy coat was then expelled into another 15 ml sterile conical centrifuge tube and diluted with 5 ml of modified RPMI 1640. Specimens were centrifuged at 300 x g for 10 minutes. The media was poured off and the cells were resuspended in another 5 ml of modified RPMI 1640. The specimens were again centrifuged at 300 x g and the media was poured off. Cells were then resuspended in RPMI complete media (10% fetal calf serum, 50 uM 2-mercaptoethanol/ml, 100 ug penicillin/ml, 100 ug streptomycin/ml, 2 uM L-glutamine/ml). Cells were resuspended and 25 ul was diluted 1:20 in 3% acetic acid. Cells were counted in a Neubauer hemacytometer. RPMI complete medium was added to each specimen to give a final concentration of 1 X 10^6 cells per ml. Then 200 ul of the cell suspension was added to each well in a row of a flat-bottom 96 well tissue culture plate (Becton Dickinson, Lincoln Park, New Jersey). The first three wells of all specimens received 10 ul of 100 ug/ml of McCoy-grown GPIC stock. The next 3 wells received 10 ul of 10 ul/ml of chlamydial HSP 60, (a kind gift from Dr. Patrick Bavoil, Rochester University, Rochester, New York). The next three rows received 10 ul of 50 ug/ml of concanavalin A (Sigma Co., St. Louis, Missouri). The last 3 wells received no antigen. Plates were incubated in 5% CO_2 for 4 days and were then pulsed with 50 ul of

20 uCi/ml of tritiated thymidine (ICN Radiobiologicals, Irvine, California). Cells were harvested 24 hours after pulse with a Packard Model 198 Cell Harvester. Radioactive thymidine uptake was read on a Packard Model Matrix 96 Direct Beta Counter. The average of 3 triplicate wells was used as the actual specimen count. The stimulation index was calculated as the specimen count divided by the average count from the 3 unstimulated wells.

Immunoblot Analysis

Immunoblot of GPIC antigens has been described previously (3). Approximately 35 ml of a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide separating gel (Bio-Rad Laboratories, Richmond, California) was poured into a 16 cm by 18 cm glass plate sandwich on a Hoefer Sturdier Model SE400 Vertical Slab Gel Unit, leaving a 3.5 cm space at the top. One ml of saturated butanol was carefully layered on top to create a sharp interface. The gel was allowed to polymerize overnight. The butanol was poured off and the top of the gel was rinsed with H_2O. A 3% polyacrylamide stacking gel was poured on top of the separating gel and a comb was carefully inserted into the stacking gel to provide a well for standards and GPIC proteins. This was allowed to polymerize 1 hour and the comb was removed. After rinsing with H_2O, 10 ul of prestained protein standard (Bio-Rad Laboratories, Hercules, California) was placed in the standard well of the stacking gel. GPIC proteins were pretreated by boiling for 2 minutes in an equal volume of 10% SDS and phenol red tracking dye. Approximately 1000 ug of treated GPIC protein was added to the protein well. Electrophoresis buffer (0.025 M tris, 0.192 M glycine, 0.1% SDS) was carefully added to the top and bottom of the vertical slab gel unit. Electrophoresis was initiated at 20 mA DC until the dye front reached the separating gel. At that time the current was increased to 25 mA. Electrophoresis was halted approximately 5 hours later when the dye front neared the bottom of the gel. The

vertical slab unit was disassembled and the gel was removed from the glass sandwich and placed on top of a nitrocellulose hybridization transfer membrane (MSI, Westboro, Massachusetts). Transblot filter paper (Bio-Rad Laboratories, Richmond, California) was placed on both sides of the nitrocellulose/gel forming a sandwich. A blot sponge was placed on either side of this and a grid was attached to both sides of the sandwich. The entire sandwich was placed in the blot tank and the tank was filled with blot tank buffer (0.025 M tris, 0.2 M glycine, 5.3 M methanol). The proteins were transferred from the gel to the nitrocellulose paper at 14 V overnight. The apparatus was disassembled and the nitrocellulose was blocked in tris-10% horse serum for 1 hour at room temperature. Three mm strips were cut, sealed in long glass tubes and stored at -20C until used. Strips were thawed at room temperature immediately before testing. Then 40 ul of thawed plasma was diluted in 3 ml of tris buffer (1.0 mM tris, 0.3 M NaCl) with 10% horse serum. This dilution was poured into the glass tube containing the nitrocellulose strip and rocked for 2 hours at room temperature. The strip was then washed 3 times with 0.155 M NaCl. Peroxidase labeled rabbit anti-guinea pig IgG was diluted 1:3000 in tris buffer-10% horse serum. Three mls were added to each specimen. Specimens were rocked at room temperature for 1 hour and washed 3 times with 0.155 M NaCl. Three ml of color developer (1.5 mg 4-Chloro-1-napthol dissolved in 0.5 ml methanol, 10 ul 3% H_2O_2, 2.5 ml tris buffer) was added to each specimen. Specimens were rocked at room temperature until distinct bands were seen, approximately 5 to 10 minutes. Strips were washed 2 times with H_2O and air dried. Molecular weights of GPIC proteins were determined by comparing bands to prestained proteins of known molecular weights contained in the protein standard.

RESULTS

Initially the course of chlamydial genital infection in male guinea pigs was evaluated. One group of 15 male Hartley strain guinea pigs was infected by urethral inoculation of 2×10^6 inclusion forming units (IFUs) of GPIC. This first group served as the initial experimental group while a second was treated in an identical manner and served as a repeat experimental group to verify the initial results. Cultures were collected at 3 day intervals until no organsisms were recovered on two consecutive specimens. The mean number of IFUs recovered for each of the 15 animals in the initial study group was plotted against the day of collection to determine the course of the infection (Figure 1). The group that was initially infected demonstrated an average of only 10^2 organisms on day 3. By day 6 post infection, the average number of IFUs rose rapidly to 3.2×10^4. At day 9, the number of IFUs recovered peaked at 6.2×10^4 and dropped off rapidly. By day 21, no organisms were recovered and the infection had resolved. Cultures collected on day 24 were also negative (not shown). Not surprisingly, there was considerable variation in the numbers of IFUs recovered from each animal as the standard deviation error bars indicate.

When the experiment was repeated with the second group of 15 guinea pigs similar results were obtained (Figure 2). At day 3, an average of 1.2×10^3 IFUs was recovered. Again the numbers rose rapidly on day 6 to 2.9×10^3. The shedding of organisms peaked on day 9 at 6.1×10^4 and then declined rapidly thereafter. Again by day 21, no organisms were recovered. Interestingly, not as much individual variation was observed in this experiment as the error bars indicate.

To determine whether animals were immune to reinfection, one group of 5 animals from the initial experimental group was inoculated in an identical manner

Figure 1.

Mean number of IFUs recovered form male guinea pigs after primary infection

(Experiment 1)

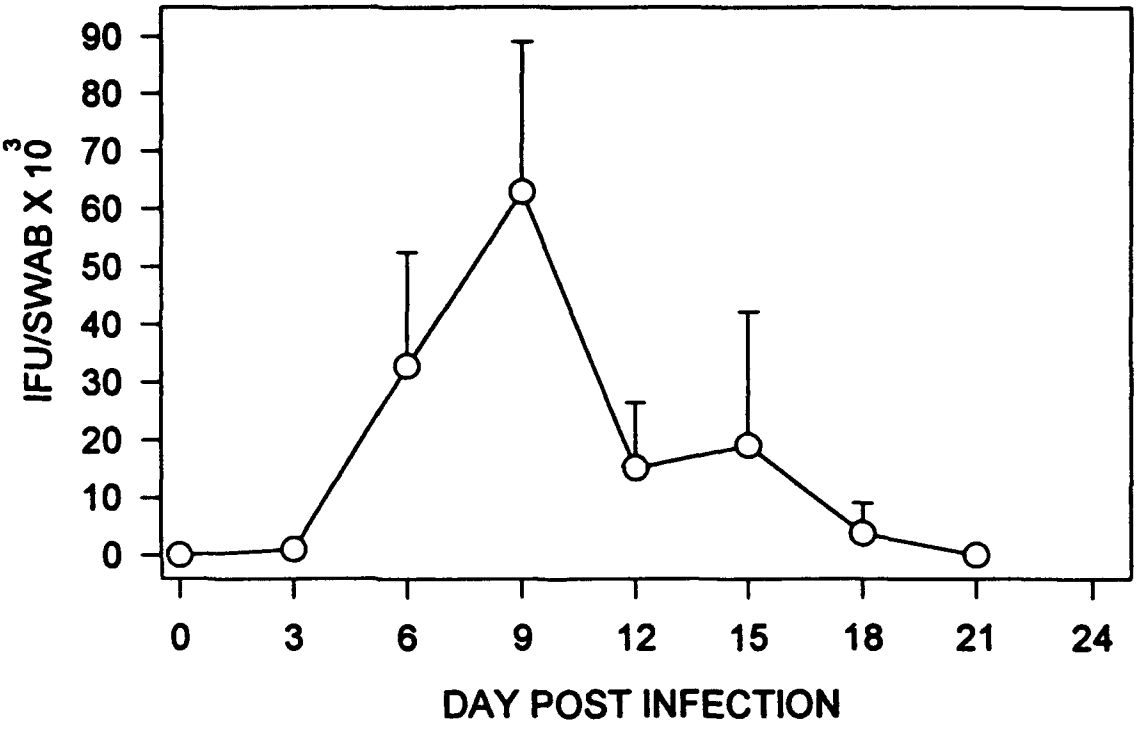
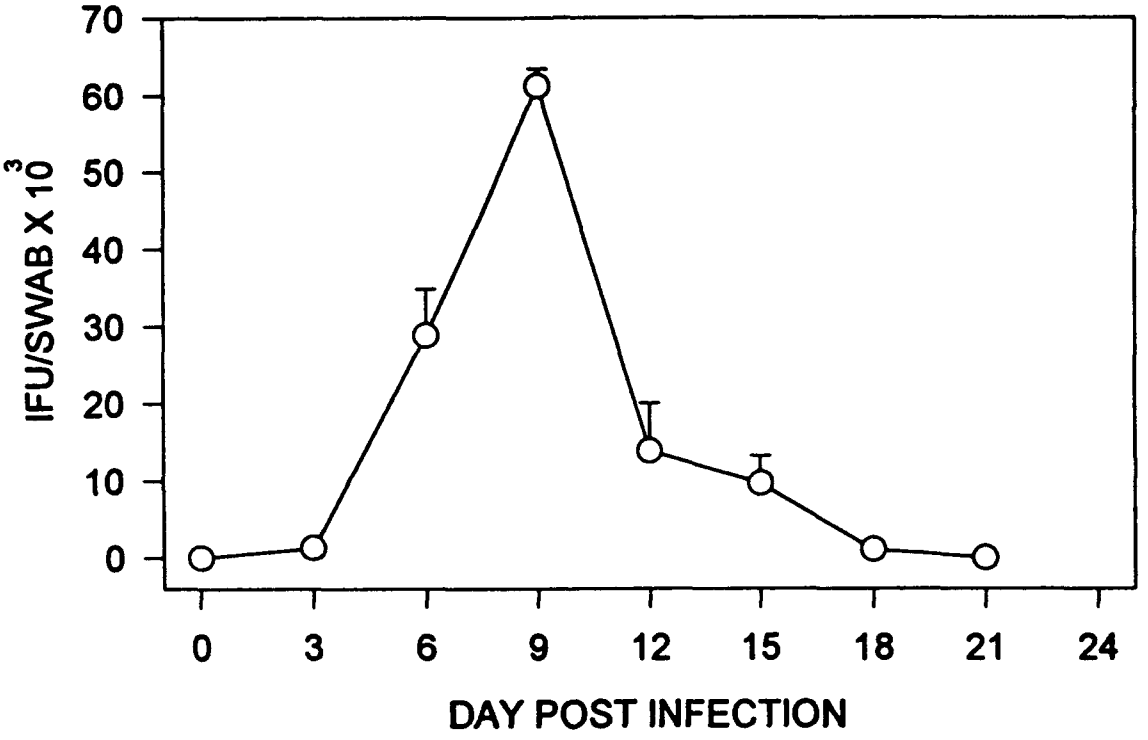

IFU/SWAB X 10^3
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
DAY POST INFECTION

Figure 2.

Mean number of IFUs recovered from male guinea pigs after primary infection

(Experiment 2)


70
60
50
40
30
20
10
0
IFU/SWAB X 10^3
0
3
6
9
12
15
18
21
24
DAY POST INFECTION

30 days after the initial infection. None of the animals were positive by isolation on day 3 post challenge. On day 6, 4 x 10^2 IFUs were recovered from one animal and 15 x 10^2 IFUs were recovered from another. No organisms could be recovered from the other 3 animals. On day 9 post challenge, 8.7 x 10^3 IFUs were recovered from the latter animal (Figure 3, A). These data were significant at $p < 0.006$ using Wilcoxon Signed Rank Test.

When a second group of 5 animals from the initial experimental group was challenged 75 days after initial infection, none of the animals shed organisms throughout the sampling period (Figure 3, B). These animals demonstrated a marked degree of immunity ($p < 0.031$)

A final group of 5 male guinea pigs was challenged 150 days after initial infection to evaluate resistance to infection. Throughout the collection period, only one animal shed 160 organisms on day 6 (Figure 3, C). No other organisms were recovered in any of the animals ($p < 0.031$). It is quite interesting that even after 150 days, the degree of immunity to reinfection was quite high. Again, the infection was very brief and of extremely low intensity when compared to the initial infection.

This entire experiment was repeated using groups of 5 guinea pigs from the second group of 15 that was initially infected. Five animals were challenged 30 days after the initial infection and cultures were collected at 3 day intervals post challenge. No organisms were recovered from any of these animals (Figure 4, A). While 100% of these animals were initially infected, none became reinfected upon 30 day challenge ($p < 0.031$).

The 75 day challenge experiment was repeated with a group of 5 animals from the second experimental group. Only one animal was positive (6 x 10^2 IFUs) on day 6. No organisms were cultured from this animal thereafter (Figure 4, B).

Figure 3.

Percent of male guinea pigs infected after primary challenge inoculation with GPIC (Experiment 1) A. 30 day challenge. B. 75 day challenge. C. 150 day challenge

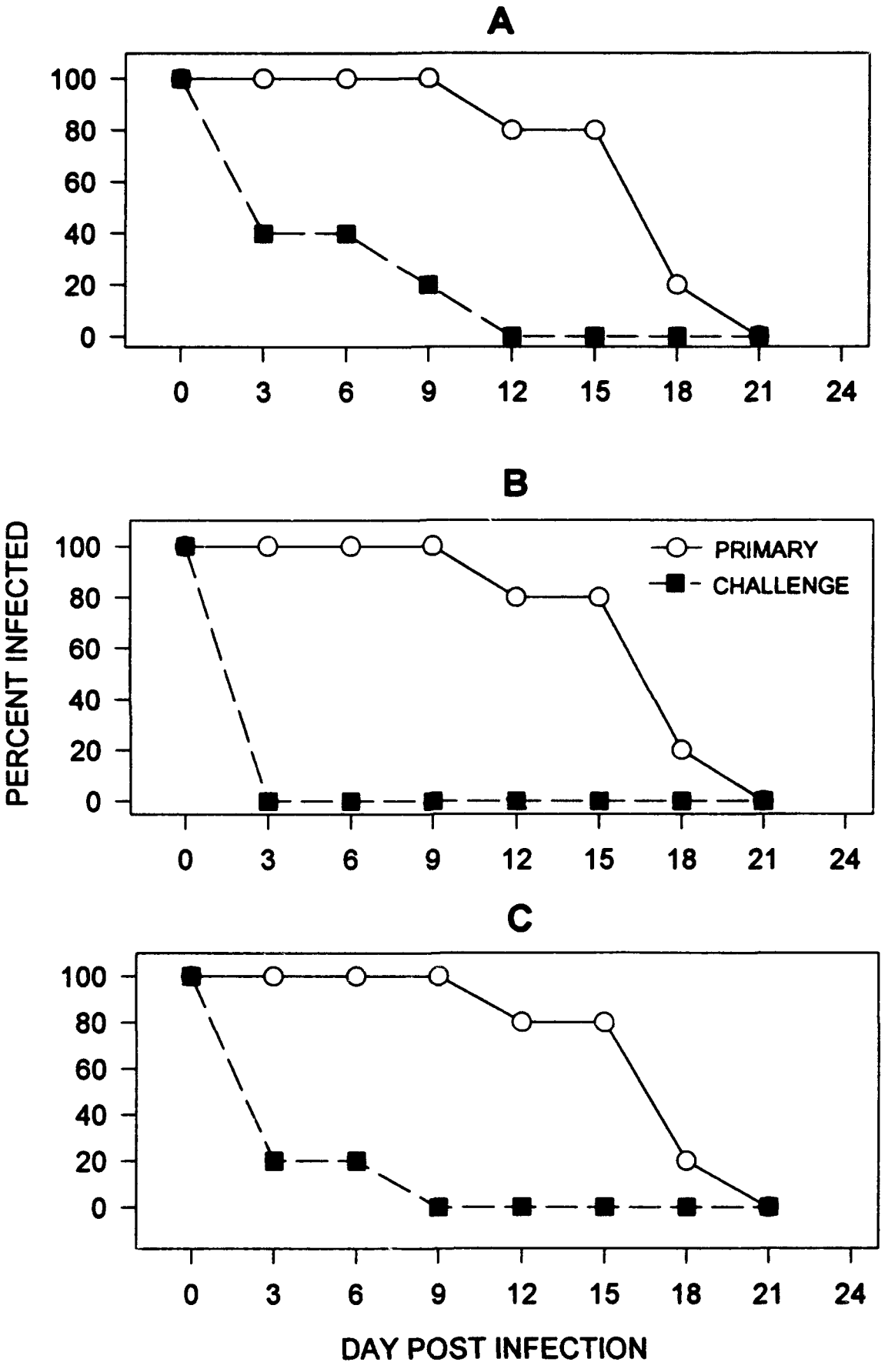
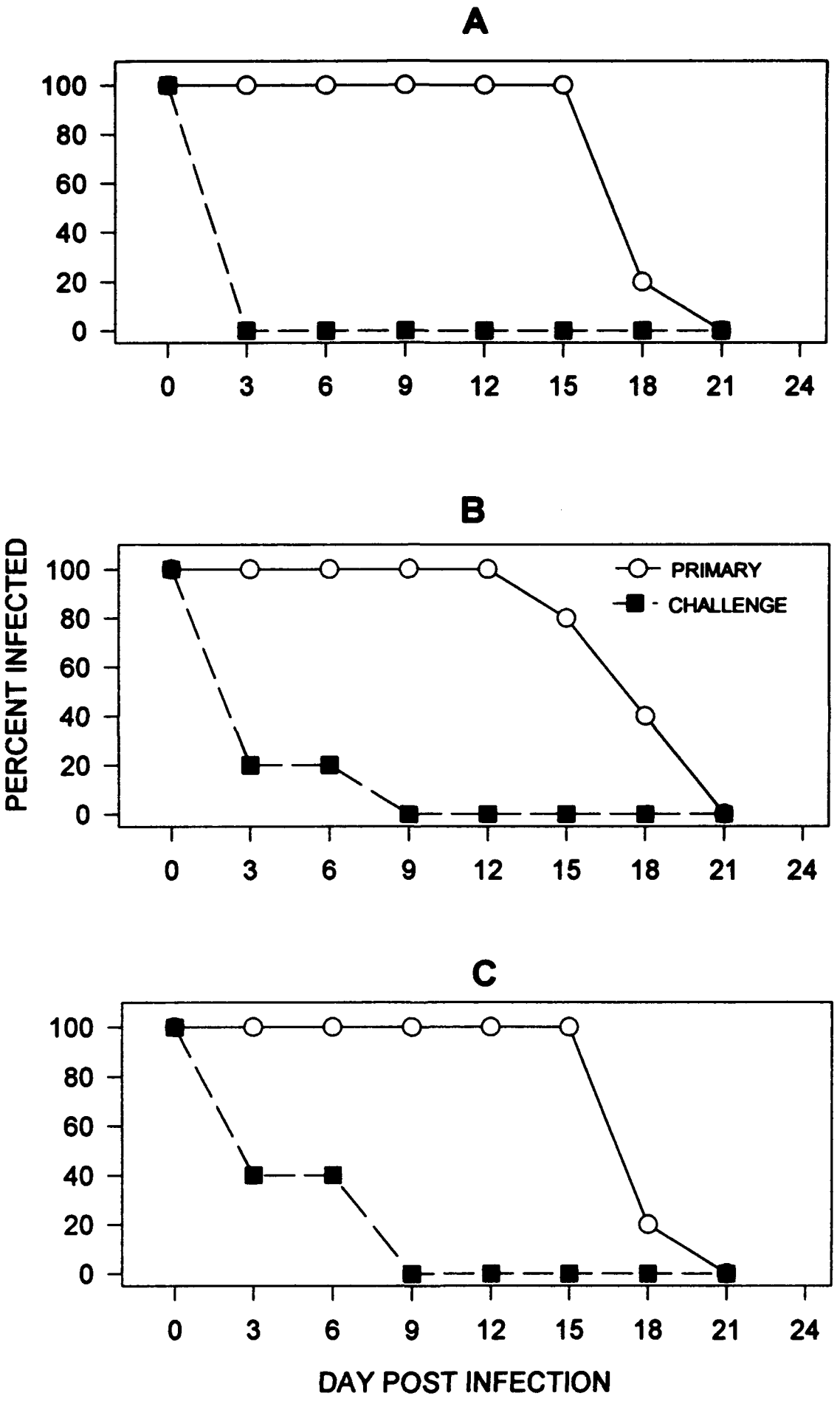

A
B
C
PERCENT INFECTED
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
PRIMARY
CHALLENGE
DAY POST INFECTION

Figure 4.

Percent of male guinea pigs infected after primary and challenge inoculation with GPIC (Experiment 2) A. 30 day challenge. B. 75 day challenge. C. 150 day challenge


A
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
B
PERCENT INFECTED
PRIMARY
CHALLENGE
C
DAY POST INFECTION

Thus only 20% became reinfected when challenged 75 days post infection indicating a high degree of immunity was still present ($p < 0.031$).

The 150 day challenge experiment was repeated using 5 animals from the second experimental group. In this experiment, two animals became reinfected, one shedding only 245 IFUs and the other shedding 9×10^3 IFUs on day 6 post challenge (Figure 4, C). These were the only organisms recovered during this experiment. Again, while all animals (100%) were intitially infected, only 2 (40%) became reinfected ($p < 0.0093$).

In order to determine the kinetics of the antibody response, one group of 5 guinea pigs was infected by urethral inoculation with GPIC. Plasma from each animal was collected at 7 day intervals post infection for 49 days and at increasing intervals thereafter for one year. When measured by enzyme linked immunosorbent assay (ELISA), the mean of the anti-GPIC IgG titer for all 5 animals was found to rise sharply to day 49 post infection (Figure 5). The titer remained fairly stable at 2.8 (log 10) until day 147 when it began to decline slightly. The titer was still elevated 365 days after infection.

The anamnestic antibody response resulting from the 30, 75, and 150 day challenge infections was also examined. The 5 animals from the first experimental 30 day challenge group were used to monitor antibody titer response to challenge infection. Plasma was collected at 7 day intervals post infection for 28 days. Specimens were also collected at 7 day intervals for 21 days after challenge. The mean titer of the log of the antibody response of the 5 animals rose until day 28 (Figure 6, A). When challenged on day 30, the titer continued to rise until day 40. The titers of the initial infections peaked at 28 days at a mean titer of 2.1 while after challenge, it rose to and stabilized at 2.7. It was assumed then that the 30 day challenge boosted the titer somewhat.

Figure 5.

Mean anti-GPIC titers in male guinea pigs after primary infection

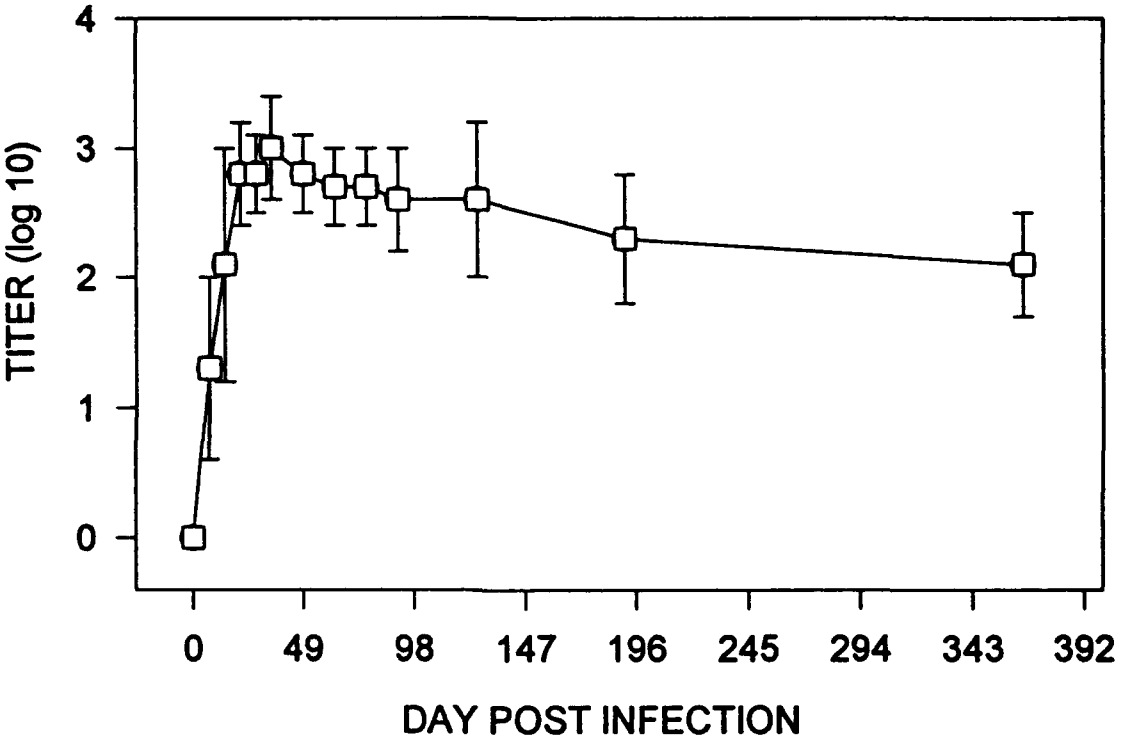

4
3
2
1
0
TITER (log 10)
0
49
98
147
196
245
294
343
392
DAY POST INFECTION

Figure 6.

Mean anti-GPIC titers in male guinea pigs after primary and challenge inoculation. (Experiment 1) A. 30 day challenge. B. 75 day challenge. C. 150 day challenge (Challenge times are indicated by breaks in horizontal axis)

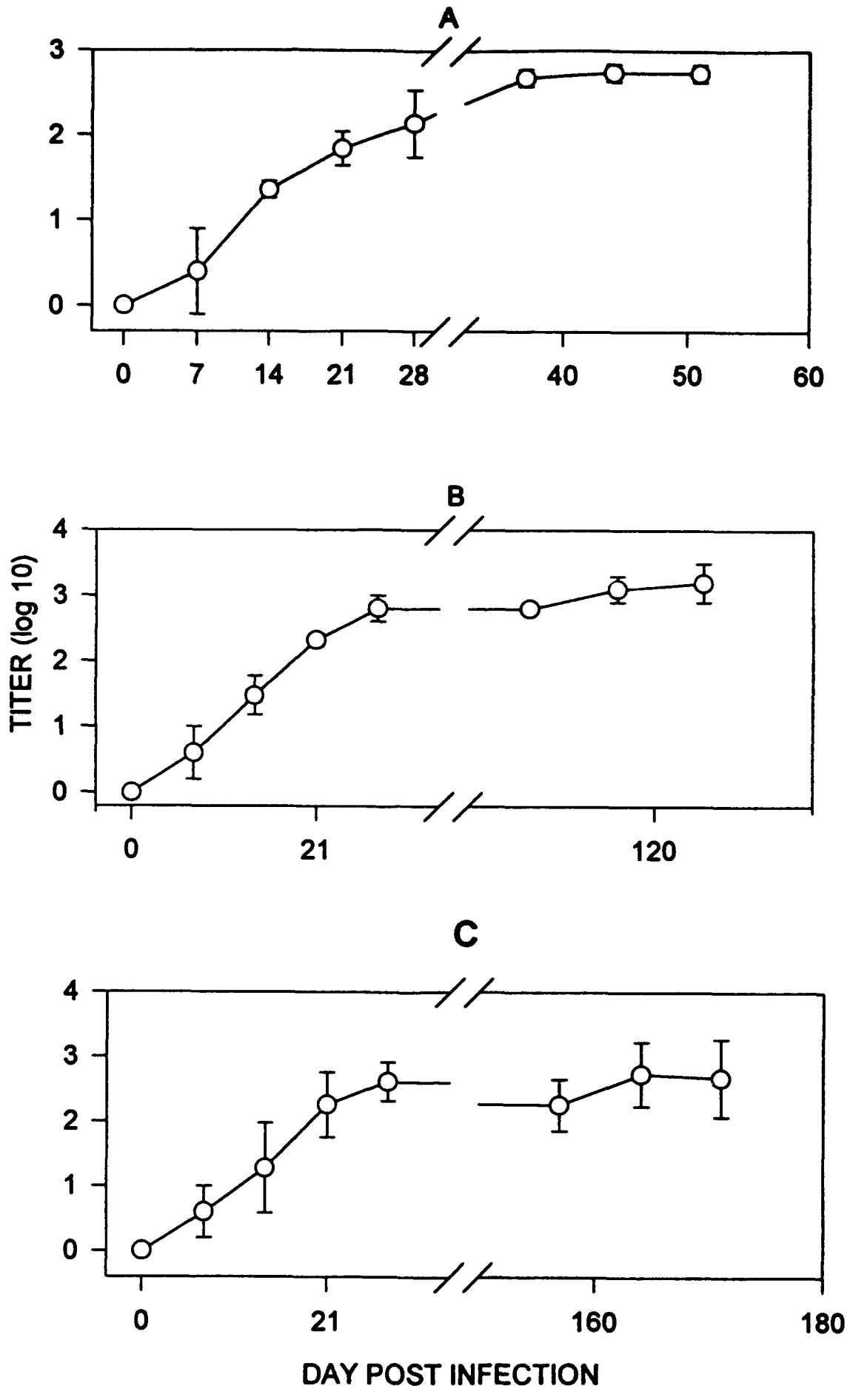
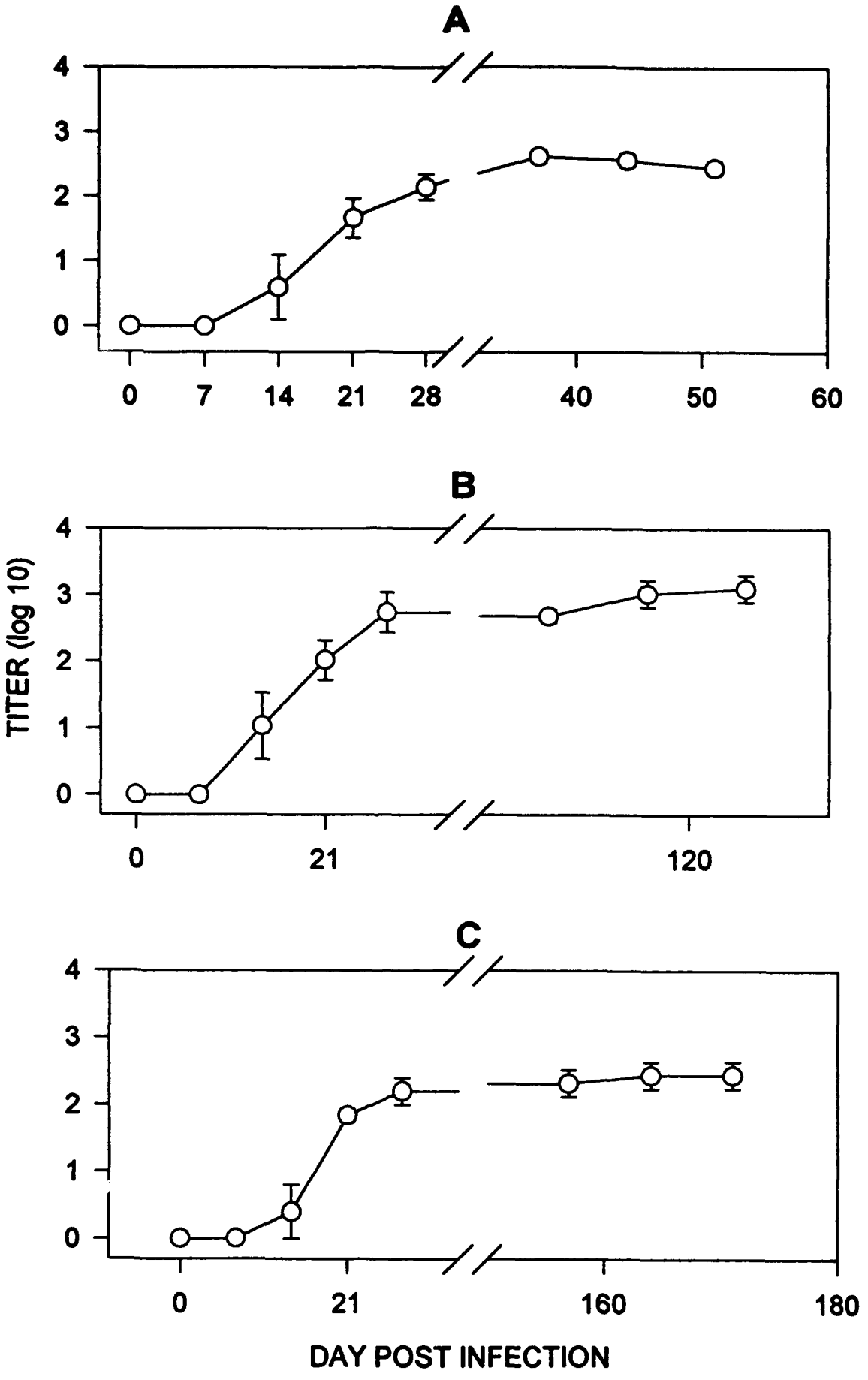

A
3
2
1
0
0
7
14
21
28
40
50
60
B
TITER (log 10)
4
3
2
1
0
0
21
120
C
4
3
2
1
0
0
21
160
180
DAY POST INFECTION

Figure 7.

Mean GPIC titers in male guinea pigs after primary and challenge inoculation. (Experiment 2) A. 30 day challenge. B. 75 day challenge. C. 150 day challenge (Challenge times are indicated by breaks in horizontal axis)


A
4
3
2
1
0
0
7
14
21
28
40
50
60
B
TITER (log 10)
4
3
2
1
0
0
21
120
C
4
3
2
1
0
0
21
160
180
DAY POST INFECTION

This experiment was repeated using the 5 animals from the initial experimental group that were challenged 75 days post infection. The results were very similar to that of the 30 day challenge group. There was a gradual rise in titer until day 28 at which time the average log of the titer leveled off at 2.8 (Figure 6, B). The challenge infection at day 75 resulted in a titer of 3.1 by day 21 post challenge (124 days after initial infection).

Five animals from the initial experimental group were also challenged 150 days after primary infection. The mean log of the antibody titer rose to 2.6 by day 28 (Figure 6, C). Upon challenge, the titer was boosted to 2.7 by day 14 post challenge (day 164 post infection).

This entire experiment was repeated using the 5 animals from the 30 day challenge group that were used in the second set of experiments. At day 21, the mean antibody titer peaked at 2.1 (Figure 7, A). Challenge infection boosted the titer to 2.6 on day 7 post challenge (day 37 post infection). The titer remained fairly stable thereafter.

This experiment was repeated using 5 animals from the second experimental group that were challenged 75 days after primary infection. As expected, titers rose to 2.7 by day 28 post infection (Figure 7, B). There was a slight boost to the titer 14 days post challenge (117 days post infection).

Again, the 150 day challenge experiment was repeated. The same pattern was noted except that the overall titers were slightly lower, peaking at 2.2 on day 28 post infection (Figure 7, C). The challenge boosted the titer to 2.4 on day 14 post challenge (day 164 post infection).

To determine the antigen specific antibody response, plasma specimens from 8 randomly selected guinea pigs were assayed by immunoblot analysis. The antibody response of animal 7909 was representative of most of the animals (Figure 8). A strong response was noted to the 60 kDa outer membrane protein

Figure 8.

Immunoblot of plasma anti-GPIC IgG in male guinea pig (7907) after primary infection and 150 day challenge infection

7907

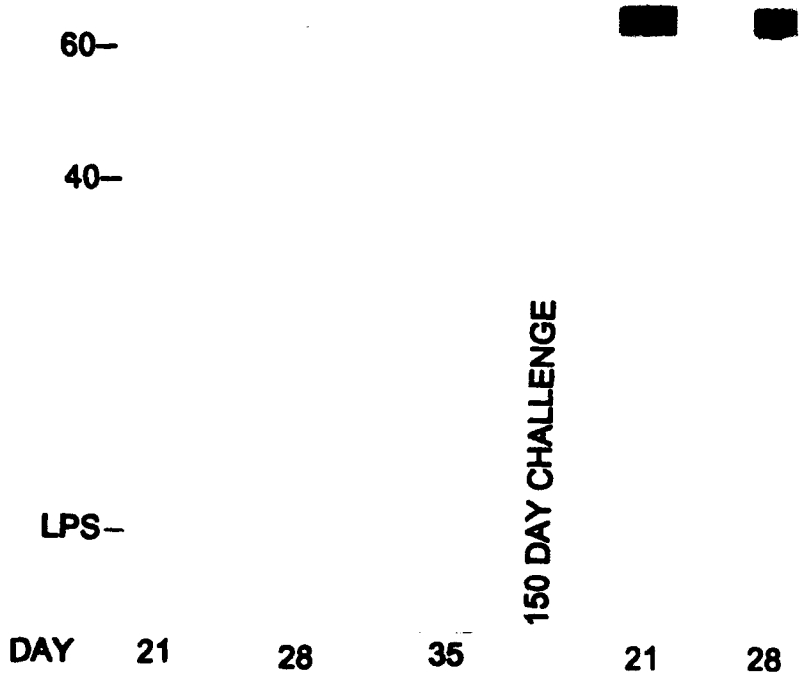
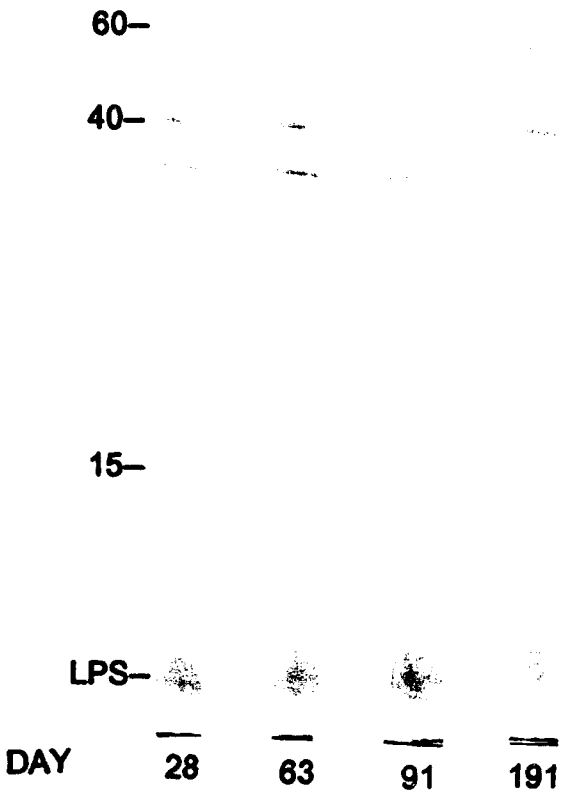


Figure 9.

Immunoblot of plasma anti-GPIC IgG in male guinea pig (7551) after primary infection

7551



(omp 2) and/or the chlamydial HSP60 protein. Antibody to these proteins demonstrated a more intense reaction upon challenge infection at 150 days. No response to the 40 kDa MOMP was noted. There was also a very weak response to LPS that cannot be seen on this reproduction.

Animal 7551 was monitored for 191 days. This animal responded to the omp2/HSP60 proteins and quite well to LPS (Figure 9). Antibodies to MOMP were also seen. Only one other animal demonstrated a very weak response to MOMP. There was also some response to other proteins that can been seen below the 60 kDa band. A response was also seen to a 15 kDa chlamydial protein.

Cell mediated responses were determined by peripheral mononuclear blood cell (PMBC) blast transformation assay. A group of 5 guinea pigs was infected as previously described. PMBCs were collected by cardiac puncture at 7 day intervals post infection. Whole GPIC and chlamydial HSP60 were used as antigens. The mean stimulation index for the 5 animals was plotted against time. It can be seen that the cell mediated response to GPIC began to rise by day 14 post infection (Figure 10). The response gradually increased until day 28 with a stimulation index of 33. Because some animals responded much better than others, there was some variation in the stimulation indices among the various animals, especially as the indices increased. The response to chlamydial HSP60 gradually increased also but this response peaked at day 21 with a stimulation index of 29. After day 21, the response fell rapidly to 4 by day 28 post infection.

This experiment was repeated with a new group of 5 male guinea pigs. The response in this group was less intense than that found in the original group. The mean response to GPIC began to rise on day 14 post infection and increased to only 16 on day 28 (Figure 11). The stimulation index to the chlamydial HSP60

Figure 10.

Mean stimulation index response for whole GPIC and HSP60. (Experiment 1)

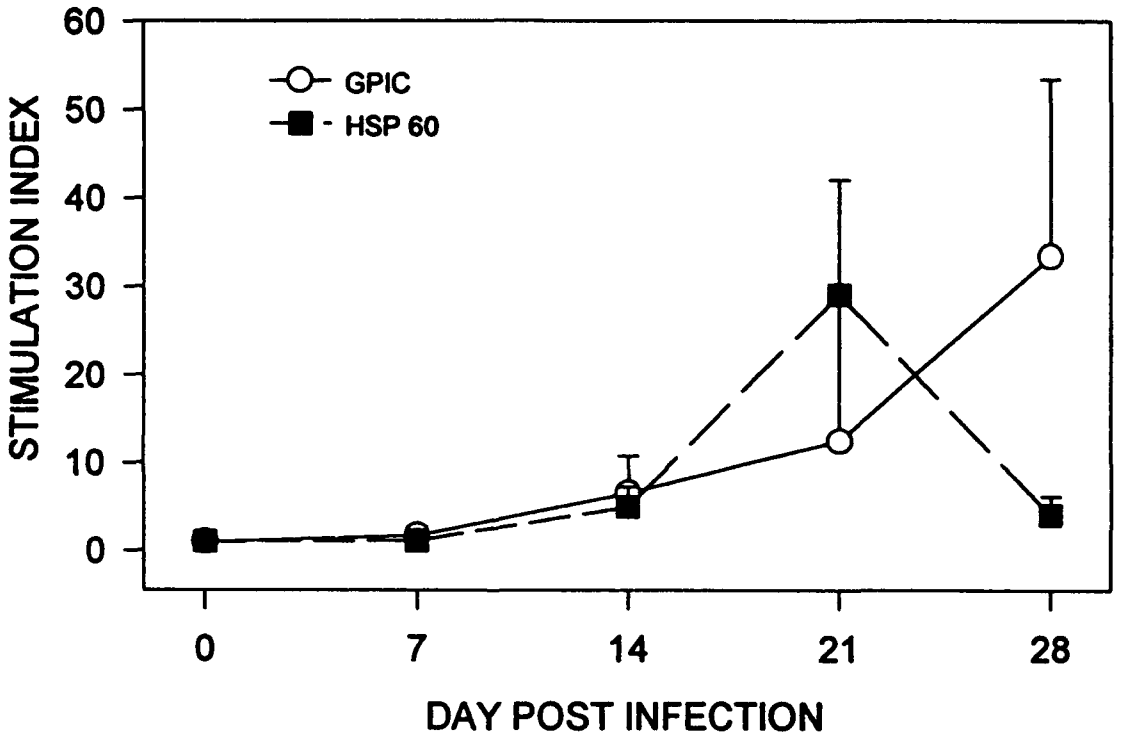
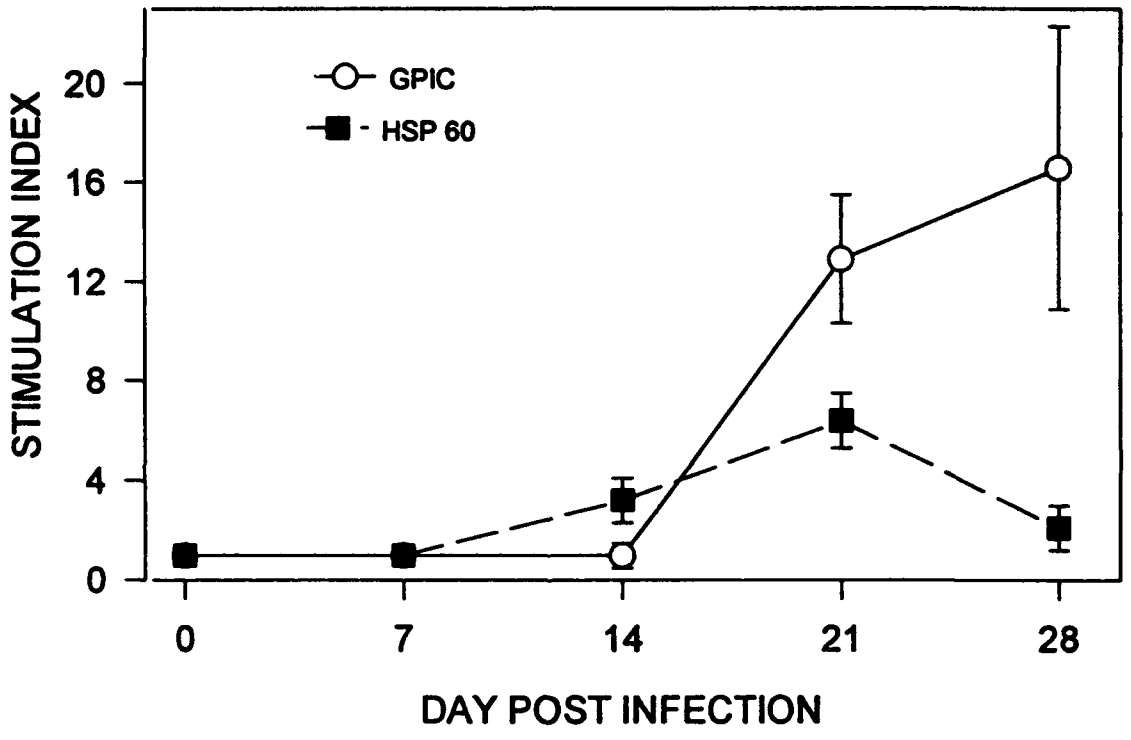

60
50
40
30
20
10
0
STIMULATION INDEX
GPIC
HSP 60
0
7
14
21
28
DAY POST INFECTION

Figure 11.

Mean stimulation index response for whole GPIC and HSP60. (Experiment2)


GPIC
HSP 60
STIMULATION INDEX
20
16
12
8
4
0
0
7
14
21
28
DAY POST INFECTION

peaked at 6 on day 21 and, like the response in the previous experiment, fell rapidly thereafter.

An important part of this study was to determine if immunization of male guinea pigs could prevent or lessen the severity and duration of a chlamydial genital infection. Five male guinea pigs were immunized subcutaneously with 100 ug of UV inactivated whole elementary bodies of GPIC. The immunization series consisted of a primary immunization and two booster immunizations at 2 week intervals. Another group of 5 male guinea pigs was not immunized and served as a control. Both groups were inoculated with 2.0×10^6 IFUs of GPIC. Urethral cultures were collected at 3 day intervals post infection until two consecutive negative cultures were obtained. All of the control animals became infected and displayed the typical course of a primary infection (Figure 12). The infection began slowly at day 3 as expected and peaked at 1.05×10^5 on day 9 post infection and then declined until the infection was resolved by day 21. The standard deviation is indicated by the error bars. In contrast, the immunized animals demonstrated a completely different response. In these animals the recovery of *Chlamydia* peaked at day 3 at 3.0×10^4 IFUs and fell rapidly until day 12 at which time no organisms could be recovered. It must be noted that one animal was completely immune to infection and never shed organisms during the course of this experiment. These data are significant at $p < 0.0001$ according to a 2-way (days, group) analysis of variance with repeated measures on one factor (days).

This experiment was repeated with additional animals to verify results. The unimmunized control group had a peak mean recovery of 8.8×10^4 IFUs at day 9 post infection. The recovery of *Chlamydia* declined until day 18 at which time no organisms were recovered. The immunized group showed a mean peak recovery of 4.3×10^4 by day 3 post infection (Figure 13). Again, one animal could not be

Figure 12.

Mean number of IFUs recovered from immunized and unimmunized male guinea pigs inoculated with GPIC (Experiment 1)

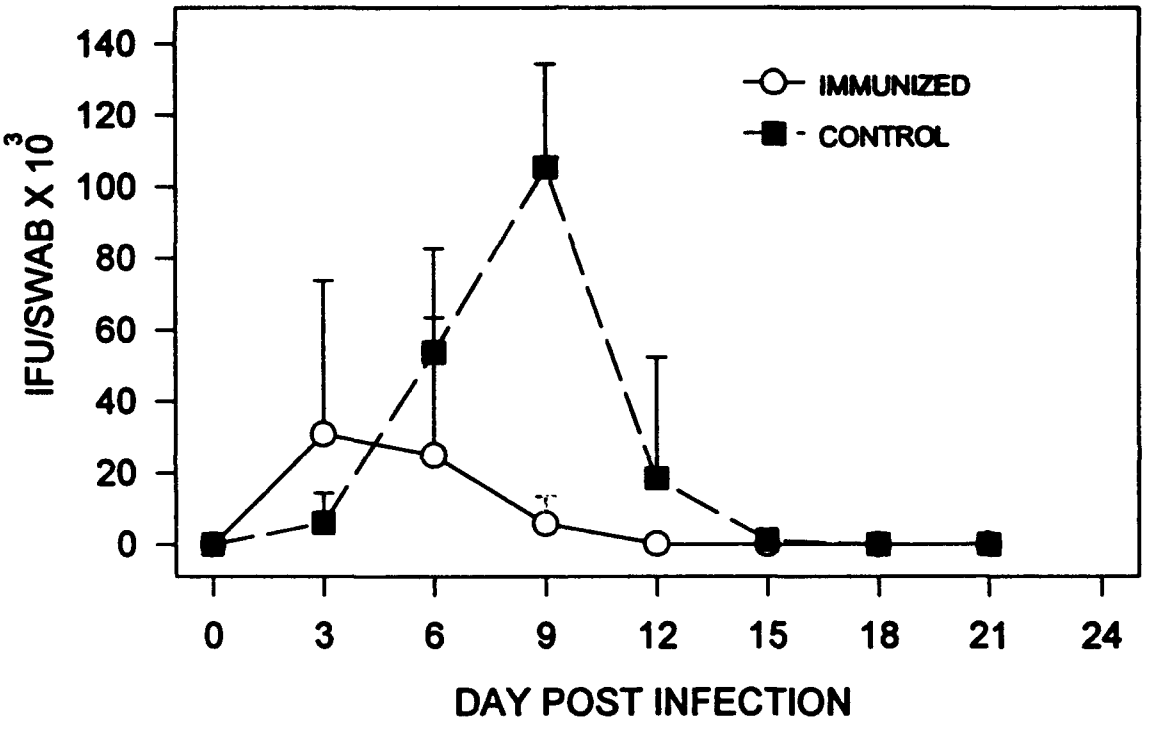
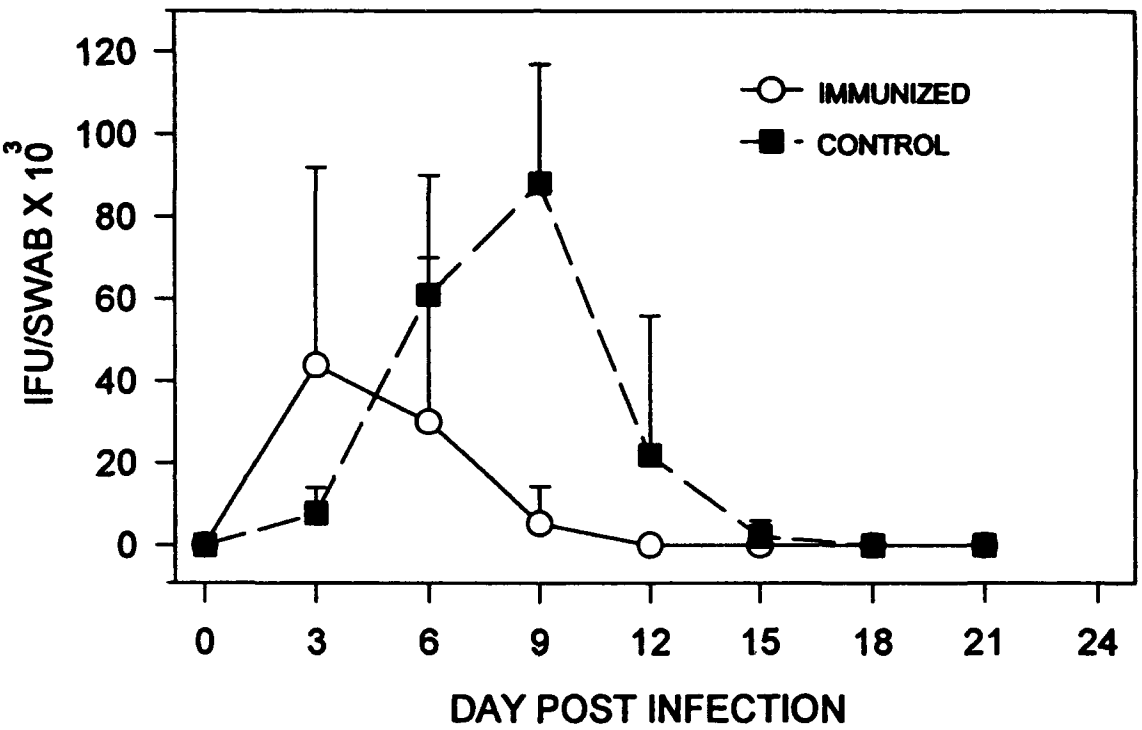

IFU/SWAB X 10^3
140
120
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
DAY POST INFECTION
IMMUNIZED
CONTROL

Figure 13.

Mean number of IFUs recovered from immunized and unimmunized male guinea pigs inoculated with GPIC (Experiment 2)


IFU/SWAB X 10^3
120
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
DAY POST INFECTION
IMMUNIZED
CONTROL

infected and never shed organisms. Only 2 animals were infected on day 12 with 82 IFUs and 240 IFUs respectively. Only one animal remained infected by day 15 ($p<.0.0001$)

The percentage of the number of animals infected throughout the course of the first experiment is depicted in Figure 14. All animals in the unimmunized control group were infected until day 15 and all had resolved the infection by day 18. Four animals (80%) of the immunized group were infected. By day 12 post infection, all had resolved the infection. These data were significant at $p < 0.0331$ according to a Wilcoxon Signed Rank Test.

The results of the second experiment are depicted in Figure 15. Again 100% of the animals in the unimmunized control group were infected and all had resolved the infection by day 18 while 80% of the immunized animals became infected. All of these animals had resolved the infection by day 18 also ($p< 0.0625$).

To determine if the immunization procedure was eliciting an antibody response, plasma specimens were collected at 7 days intervals post infection from immunized animals from the initial immunization experiment. Specimens were also collected from the unimmunized control group for comparison. All specimens were analyzed by ELISA for anti-GPIC IgG antibody. The unimmunized control animals demonstrated a typical mean antibody curve, gradually rising to day 28 (Figure 16). The immunized group however, had an average mean titer of approximately 3.8 throughout the entire experiment. The immunization had obviously produced a very strong, sustained antibody response.

This experiment was repeated and the results were found to be similar. The unimmunized control group displayed the normal rise in mean antibody titer to day 28 (Figure 17). The immunized group again had a very strong and stable antibody response to GPIC.

Figure 14.

Percent of immunized and unimmunized control male guinea pigs infected after inoculation with GPIC (Experiment 1)

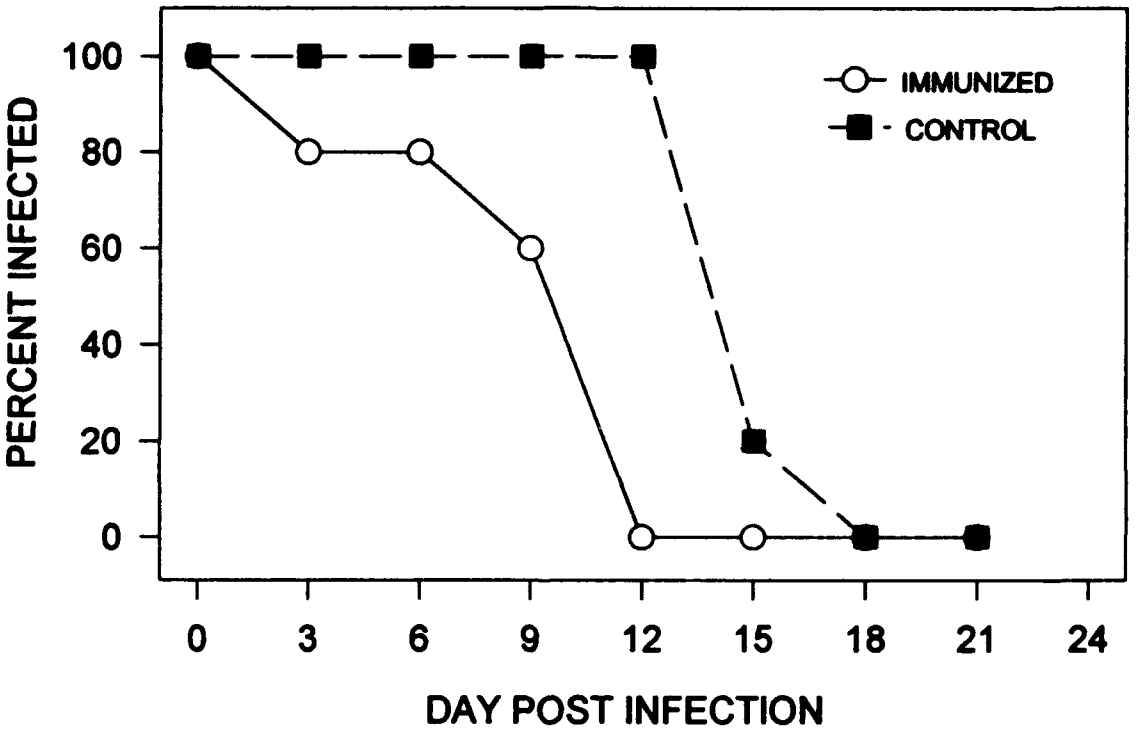

PERCENT INFECTED
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
DAY POST INFECTION
IMMUNIZED
CONTROL

Figure 15.

Percent of immunized and unimmunized control male guinea pigs infected after inoculation with GPIC (Experiment 2)

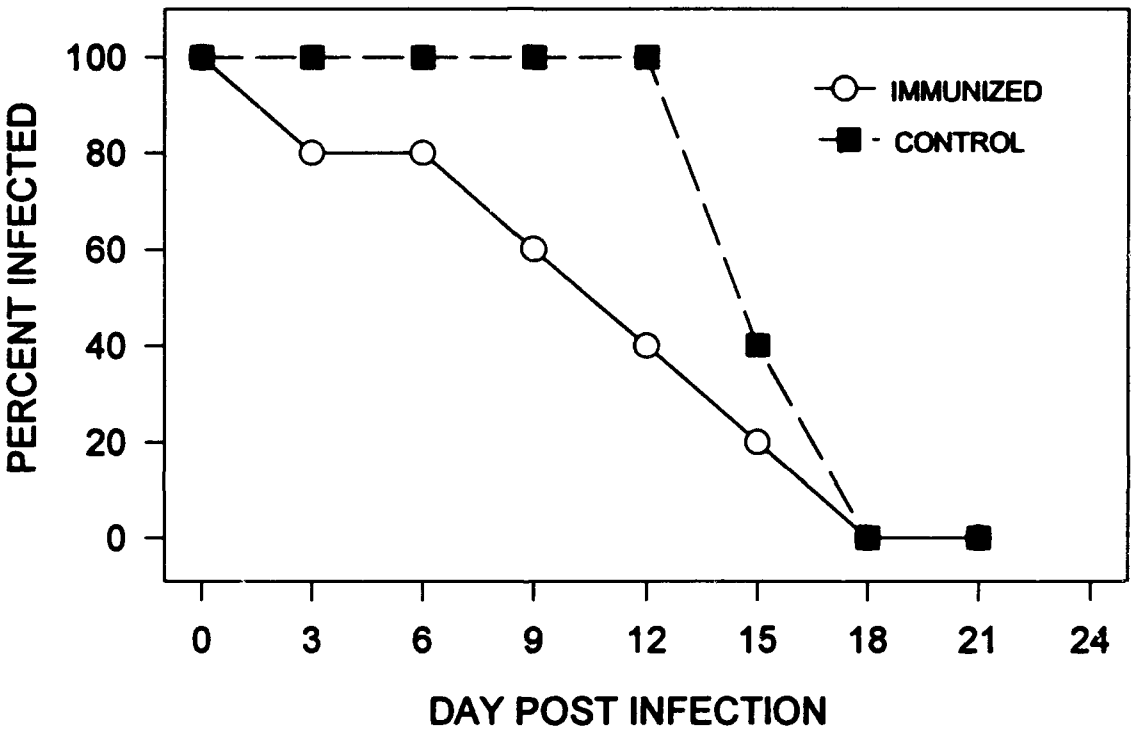

PERCENT INFECTED
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
DAY POST INFECTION
IMMUNIZED
CONTROL

Figure 16.

Mean titer of anti-GPIC antibody in immunized and unimmunized control male guinea pigs after inoculation with GPIC (Experiment 1)

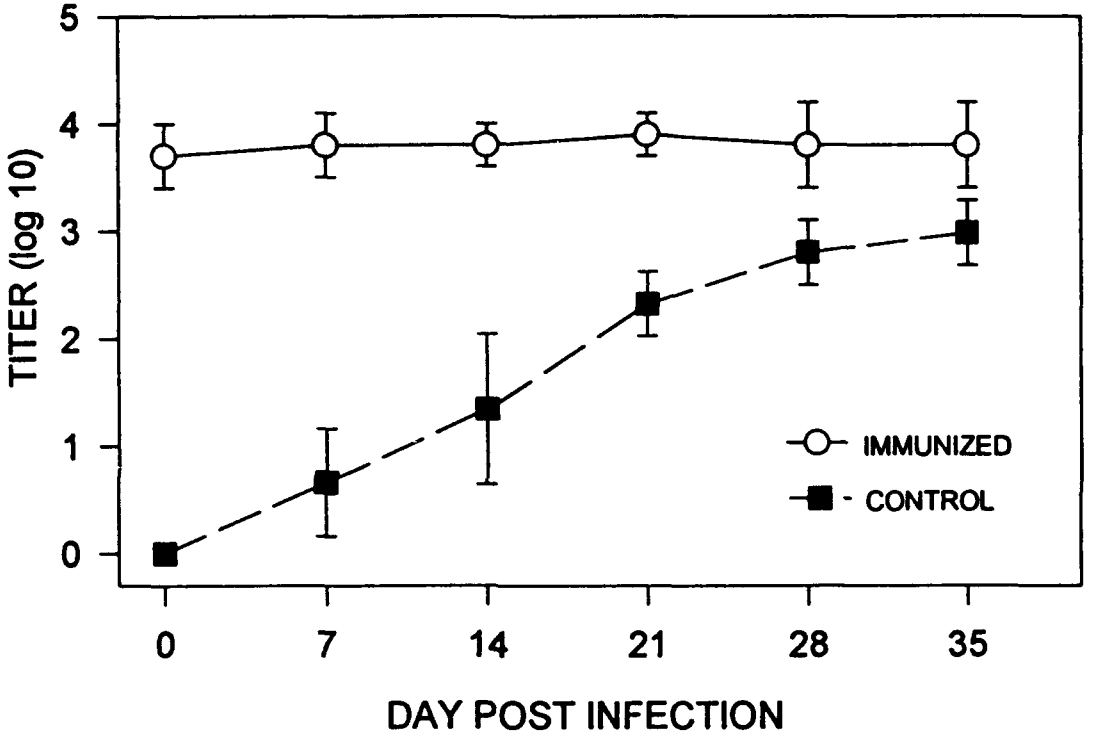

5
4
3
2
1
0
TITER (log 10)
0
7
14
21
28
35
DAY POST INFECTION
IMMUNIZED
CONTROL

Figure 17.

Mean titer of anti-GPIC antibody in immunized and unimmunized control male guinea pigs after inoculation with GPIC (Experiment 2)

DISCUSSION

The initial finding in this study was that the course of a chlamydial genital infection in male guinea pigs was similar to that found in females (36). As described in the female, the infection reached peak levels on day 9 post infection and usually resolved by day 21.

Of further interest was our observation that a primary chlamydial genital infection generated a very effective protective immune response to challenge infection in male guinea pigs. Human epidemiological studies also suggest that there may be immunity to *Chlamydia* if an individual has recently been exposed to the organism (21). However, this immunity is relatively short lived. Studies in animal models generally support this idea. Rank et. al. (35) found that female guinea pigs challenged 30 days after a primary infection were immune to reinfection. However, when challenged 77 days after initial infection, all animals became reinfected although the intensity and duration of the challenge infection were abbreviated.

In contrast, this study demonstrated that in comparison to females, male guinea pigs were remarkably immune to reinfection, even to 150 days after initial infection. The reason for this is unclear, although it appears that better protection in male guinea pigs is not due to differences in antibody titer. The kinetics of the humoral immune response in males was found to be very similar to that found in females. Anti-GPIC IgG titers rose rapidly after infection and remained elevated for at least 1 year as was described in female guinea pigs by Rank et. al. (35).

There may be a very slight difference in qualitative response but that cannot be stated with certainty at this point. Analysis of antigen-specific antibody responses revealed that all male guinea pigs in this study responded well to a 60 kDa protein (omp2 and HSP60 are both found in this band) and to LPS.

Response to the 40 kDa major outer membrane protein (MOMP) was not seen in 6 of the 8 animals tested. In contrast, Batteiger and Rank (3) found that female guinea pigs showed strong antibody responses to omp2, LPS, and MOMP, although the response to MOMP was weaker and waned with time. In one study of GPIC ocular infections in guinea pigs, the absence of a response to MOMP was also noted (49). The reason for the poor response to MOMP observed in this study is unclear but it may be due to a lack of sensitivity of the peroxidase conjugate assay. Another possibility is that the effective dose of pathogenic organisms may be higher in the female since the inoculum can remain in contact with target tissues for a much longer period of time when compared to the male where the urethra is constantly flushed during urination. Hence, a quantatively more immunogenic dose may be present in the female which may result in a stronger and broader antibody response.

A crucial point is that the absence of a response to MOMP in males would not explain the increased resistance to reinfection. If anything, it would argue against increased immune protection as antibody to MOMP has been shown to be protective in vitro (11) and in vivo (4). Aside from this, the immune response to specific chlamydial antigens appears to be essentially the same in both male and female guinea pigs.

The cell mediated response of the males in this study was also very similar to that found in females. Peripheral blood mononuclear cell responsiveness to whole GPIC began about 14 days post infection and increased until day 28 post infection. Female guinea pigs showed a similar pattern with the cell mediated response peaking at day 28 and then a gradually declining (35).

Interestingly, the male guinea pigs in this study also exhibited a cell mediated response to chlamydial HSP 60. This response peaked at day 21 post infection and then declined rapidly. It should also be noted that ponse was not as

strong as the response to whole GPIC. Studies have shown that females also respond strongly to HSP60, especially in ascending infections of the genital tract (52). How this relates to pathological changes is not known at this time.

In general, it appears that the immune response in male guinea pigs is very similar to that found in females. If there are no significant differences, perhaps the male's resistance to reinfection might be explained by a combination of immunologic and anatomical differences. That the anatomy of the genital tract might have some bearing on infection has been considered by Schachter et. al. (44). An inoculum of chlamydiae deposited in the vagina during sexual intercourse would remain trapped in the vaginal mucous for a relatively long period of time. Conditions would be ideal for large numbers of organisms to be taken up by the vaginal epithelial cells. In contrast, the male urethra has only a fraction of the surface area compared to that of the vagina. In addition, ejaculation and urination would flush organisms from the male urethra so that the inoculum would naturally be much smaller in the male.

Tissue trophism might also be a factor. *Chlamydia* can infect several types of mucosal epithelial cells but it may show greater infectivity for one cell type over another. The male urethra is lined by an unusual mucosal epithelium called stratified columnar. The vagina is lined with stratified squamous epithelium while the cervix, uterus, and fallopian tubes are lined with simple and ciliated columnar epithelium. It is not known if different epithelial tissue types play a role in infectivity but it is a possibility worth considering.

Another point to consider is that hormonal differences may play a significant role in the immune response to genital infection. Rank, et. al. (33) found that female guinea pigs treated with estradiol had significantly prolonged chlamydial genital infections. This was associated with a delay in the appearance of secretory IgG and IgA antibody induced by hormone treatment. Thus hormonal

fluctuations during the menstrual cycle may make females more prone to chlamydial genital infections. It is however, logical to assume that differences in anatomy and physiology alone do not account for the increased resistance to reinfection in male guinea pigs for they easily become infected on initial inoculation with viable organisms.

The ultimate goal of this study was to determine whether immunization of the male would be effective in preventing infection or lessening the severity and course of the infection. This study demonstrataed that male guinea pigs immunized parenterally with UV inactivated elementary bodies exhibited a marked degree of protection. Of the 2 groups tested, one from each group of 5 was completely immune to reinfection. The course of infection was abbreviated and the intensity of infection was markedly reduced in the other animals when compared to an unimmunized control group. Thus effective immunization is indeed possible but the regimen used in this study was still not able to elicit as effective a response as a natural infection.

Immunization in female guinea pigs has been partially successful. Rank et. al. (36) found that female guinea pigs immunized subcutaneously with UV inactivated GPIC were not resistant to challenge infection. The length of the challenge infection was unaltered even though high titers of antibody were present in the plasma. However, the intensity of the infection was markedly reduced when monitored by inclusion scores. As was noted in this study of the male, better protection was noted when females recovered from a natural infection.

The more effective immune response to live organisms may be due to the generation of additional antigens recently found only in live chlamydiae. Rockey et. al. (43) found that certain chlamydial protein antigens could only be obtained from cells infected with metabolically active GPIC. These additional proteins

were most likely undergoing post translational modification in viable intracellular chlamydiae. Antibody elicited by these additional antigens may provide a more effective immune response than would be found if inactivated organisms were used for immunization.

It appears from this study that male guinea pigs are more resistant to reinfection than are females. It should also noted that the parameters of humoral and cell mediated immune responses in the male animal are very similar to that found in the female in that no significant differences were observed. However, there may be differences yet to be discovered. Virtually nothing is known about mucosal or secretory immune response in the genital tissues of the male. The nature of the anatomy and physiology of the male gential tract makes these types of studies extremely difficult. It appears that the combination of immunity and male anatomy may be the key to the effective protection against reinfection that was observed in this study. If males could be immunized with an effective or partially effective vaccine, they may transmit a much smaller inoculum to the female during sexual intercourse and thus reduce the severity of the pathology associated with this disease.

SUMMARY

This study was conducted to investigate the immune response of male guinea pigs to a chlamydial genital infection. Specifically, the course and intensity of a primary infection, the course and intensity of a challenge infection, the humoral immune response, the cell mediated immune response, and response to immunization were observed.

It was found that the course of a chlamydial gential infection in male guinea pigs was similar to that found in female guinea pigs, peaking at day 9 post infection and resolving by day 21. The male guinea pigs in this study however, were remarkably resistant to reinfection at 30, 75 and even 150 days after a primary infection. Those that were infected only shed very few organisms on one or two days post challenge. Previous studies have shown that female guinea pigs were not nearly as resistant to challenge infection, especially beyond 75 days post infection even though the intensity and duration of infection were abbreviated.

The humoral immune response was very similar to that found in female guinea pigs. A strong, sustained serum anti-GPIC IgG antibody response was observed in all animals. Immunoblot analysis of antigen-specific antibody response to GPIC revealed that the male response was almost identical to that of the female. Strong responses were seen to the 60 kDa omp2/HSP60 proteins and LPS. However, there was some lack of response in males to MOMP but the reasons for this are unclear. Female guinea pigs usually respond well to this chlamydial antigen.

The cell mediated response to whole GPIC was similar to that found in females, rising to day 28. Male guinea pigs also demonstrated a significant cell mediated response to chlamydial HSP60, peaking at day 21 post infection.

Immunization provided significant protection against primary genital infection with GPIC. Male guinea pigs immunized with UV inactivated whole GPIC showed a significant reduction in intensity of a primary infection in addition to an abbreviated course of infection. However, a natural infection seemed to confer better protection than the immunization regimen used in this study.

It appears from this study that male guinea pigs are significantly more immune to reinfection with GPIC than are female guinea pigs. The reasons for this are not clear but a combination of immunity and differences in anatomy and physiology may be responsible.

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